The Characterisation Of *MAD3*: A Component Of The Spindle Checkpoint In *Saccharomyces cerevisiae*

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For Mum and Dad.

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Common abbreviations used in this work

Abbreviation used	Meaning
Amp	Ampicillin
ATP	Adenosine-5'-triphosphate
BUB	Budding uninhibited by benomyl
CTP	Cytosine-5'-triphosphate
DNA	Dioxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra acetic acid
EGTA	1,2-Di (2-aminoethoxy) ethane-N,N,N',N'-tetra acetic acid
GST	Glutathione S-Transferase
GTP	Guanine-5'-triphosphate
HA	Haemagglutinin
HRP	Horse raddish peroxidase
IP	Immunoprecipitation
Kan	Kanamycin
LB	Luria broth
MAD	Mitotic arrest difficient
MTOC	Microtubule organising centre
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBSS	PBS with Sorbitol
PBS-T	PBS with Tween-20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

Common abbreviations used in this work (Contd)

Abbreviation used	Meaning
PMSF	Phenyl methyl sulphonyl flouride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SGD	*Saccharomyces* genome database
TAE	Tris acetate EDTA solution
Tet	Tetracycline
TTP	Thiamine-5'-triphosphate
YMM	Yeast minimal media
YPDA	Yeast peptone with dextrose and adenine
YPD	Yeast peptone with dextrose

Chapter 1 - Introduction

1.1The Cell Cycle

1.1.1 The General Cell cycle of Eukaryotes

From the smallest cell to the largest animal, life consists of an eternal cycle of growth and rebirth. The smallest unit of life, the cell, grows by assimilating nutrients from the environment, be that environment the ocean or the blood stream. The ultimate goal of all organisms is to reproduce and pass on the genetic material, composed of deoxyribonucleic acids (DNA), to the next generation. To do this the organism must divide. So in growing, using the nutrients available, the cell can divide into two units with all the necessary genetic material to continue the cycle. This continuous growth and division is called the cell cycle.

Cellular life takes two basic forms, the prokaryotes (bacteria) and the eukaryotes (from the simplest yeast to us). The cell cycle of eukaryotes typically consists of two growth phases (G1 and G2), a phase where the DNA is copied (S-phase), and a division phase (Mitosis, M-phase) in the order; G1, S, G2 and M (Figure 1.1) (Molecular Biology of the Cell, Alberts *et al*, Garland Publishing, 1994). The DNA is tightly packed into structures called chromosomes, which are enclosed in a membranous organelle called the nucleus (Figure 1.1). The somatic cells of humans have two sets of the genome (a diploid organism) making 46 chromosomes in total (2 X 23 chromosomes), whereas budding yeast, in its haploid form, has only a single set on 16 chromosomes (1 X 16 chromosomes).

A signal is sent to the chromosomes once the cell has reached a critical size and the DNA is replicated. Once replicated in S-phase it now consists of two chromosomes attached to each other, these are now termed sister chromatids (Figure 1.1). There are now two copies of the genome in the cell. After a second growth phase, G2, these sister chromatids are segregated to the two daughter cells in a spectacular part of the cell cycle called mitosis (Figure 1.1 M).

Mitosis is an ingenious method by which to divide paired items equally. The tightly associated sister chromatids are positioned in a line along the centre of the cell with each sister facing a different pole (Figure 1.1 M). Once they have all aligned

Figure 1.1

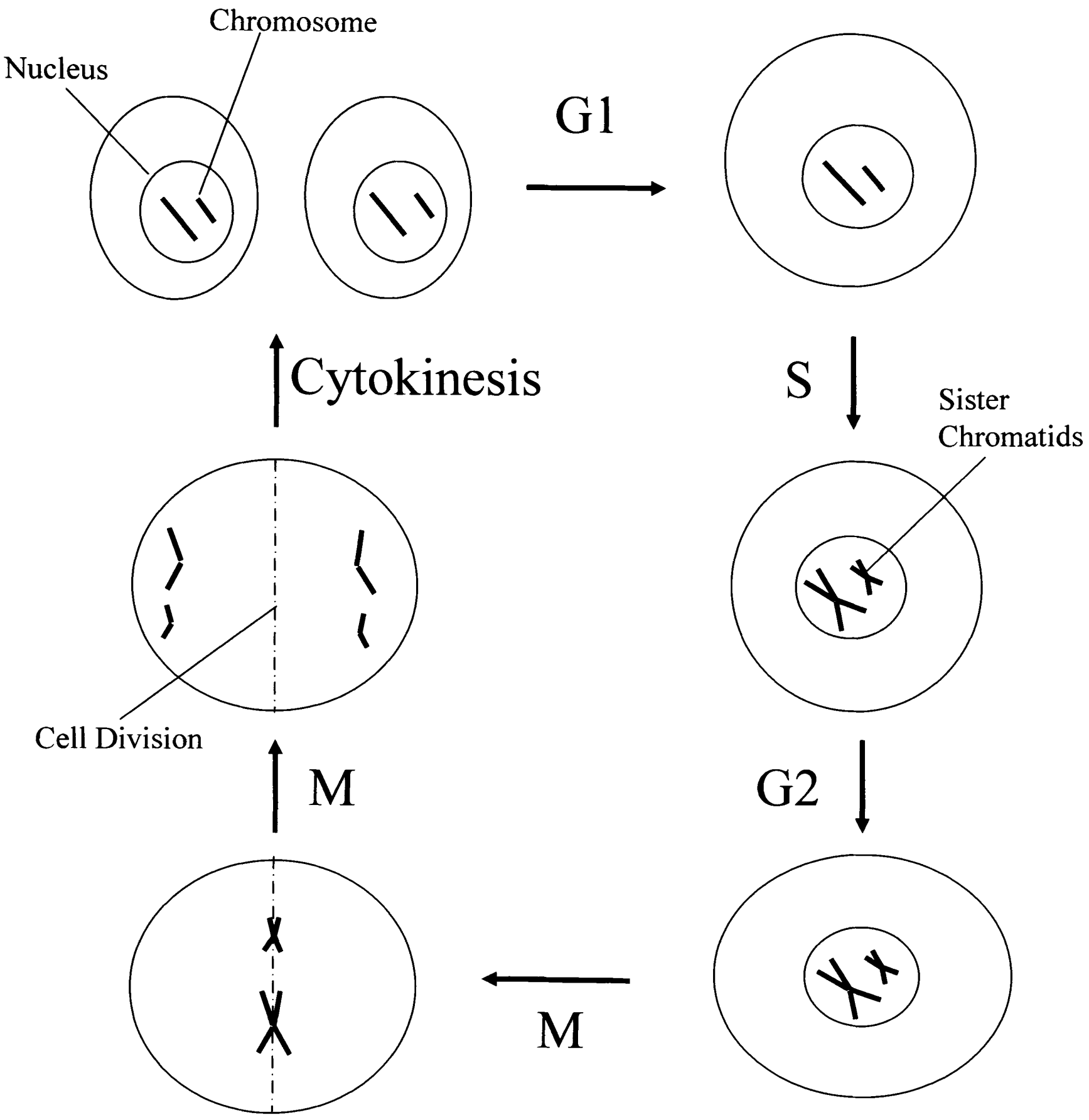


Figure 1.1 The Eukaryotic cell cycle. The cell cycle could be called the DNA cycle as it is the replication and division of the DNA which drives each part of the cycle.

correctly the cell pulls them apart and drags them towards opposite poles (Figure 1.1 M). Once the chromatids have migrated the cell is physically split in two along the line where the sister chromatids had aligned resulting in two cells, now in G1, ready to begin the cycle again. This physical separation is called cytokinesis (Figure 1.1).

It is vitally important that sister chromatid segregation (mitosis) does not proceed without the completion of DNA replication, and it follows that cytokinesis would be disastrous if it were to occur when the sister chromatids had not been segregated. To prevent these disasters the cell has evolved several levels of control. An initial level of control is provided by the cyclin dependent kinases (CDK), and their binding partners, the cyclins. A cyclin's timely association, disassociation, and degradation, initiates and drives each phase of the cell cycle. Mammalian cells have six CDK proteins and many more cyclins (Fisher 1997). This range of regulation allows the mammalian embryonic cell to divide and develop into the numerous different cells in our body. The simple budding yeast, *Saccharomyces cerevisiae*, has only one CDK (encoded by the *CDC28* gene), and nine known cyclins (encoded by *CLN1,2,3* and *CLB1,2,3,4,5,6* genes) (Mendenhall and Hodge 1998). The regulated association of Cdc28p with each cyclin links the completion of one event with the initiation of the next. In the case of budding yeast Cln1p, Cln2p and Cln3p associate with Cdc28p in G1 and initiate DNA synthesis (S-phase) (Koch, Schleiffer et al. 1996), Clb5p and Clb6p associate with Cdc28p during S-phase and ensure its completion (Amon, Tyers et al. 1993). Finally, Clb1p, Clb2p, Clb3p and Clb4p associate with Cdc28p to initiate mitosis and lead the cell cycle to its completion. The degradation of Clb2p leading the procession back into the G1 state (Lim, Goh et al. 1998).

The following sections explain the details of these transitions and their regulation.

1.1.2 The Cell Cycle of the budding yeast, *Saccharomyces cerevisiae*

This work uses the budding yeast *Saccharomyces cerevisiae* as a model organism to study the cell cycle. There are some differences in the cell cycle of budding yeast and the generalised one described above. Where necessary these differences will be explained.

Saccharomyces cerevisiae is a budding yeast and it does just that, bud. In this way it does not resemble the general cell cycle by dividing the cell equally in two but it does divide the nucleus in two. This transmits a copy of the genome (the total DNA required to make a budding yeast) to the bud and leaves one copy in the progenitor cell, termed the mother cell. Figure 1.2 shows the cell cycle of *S. cerevisiae*.

During G1 budding yeast has two choices: 1) it can enter the cell cycle and replicate the DNA, 2) it can leave the cell cycle and mate. Mating is achieved by making a mating projection (Figure 1.2 A) and forming a link with another budding yeast cell of opposite mating type (White and Rose 2001). Mating leads to the sexual cycle of budding yeast where the nuclei from two yeast of opposite mating type (either **a** or α) fuse, forming a diploid zygote which may then divide indefinitely or, under certain conditions such as starvation, enter meiosis and form spores. The mating projection forms in response to a chemical signal caused by the α-factor or a-factor proteins which are released into the medium by either sex of yeast cell. These pheromones prevent the cells from entering S-phase and induce the fomations of mating projections leading to the fusion of the two cells and ultimately the fusion of the nuclei (White and Rose 2001). The α-factor induced arrest has been used by researchers, and indeed in this work, to synchronise a culture of yeast before S-phase (Shedden and Cooper 2002).

To enter S-phase the yeast CDK, Cdc28p, associates with Cln3p (Levine, Tinkelenberg et al. 1995). This initiates the transcription of the *CLN1* and *CLN2* genes. Once activated the Cdc28p/Cln1p or Cln2p complexes trigger entry into S-phase (McInerny, Partridge et al. 1997).

The Cdc28p/Cln1,2p complexes initiates DNA replication by triggering the transcription of S-phase specific genes (Levine, Tinkelenberg et al. 1995). Ultimately this leads to the transcription of the B-type cyclins; *CLB1,2,3,4,5* and *CLB6*. The Cdc28p/Clb complexes that form, inactivate the Cdc28p/Cln3p complex freeing the catalytic subunit with its bound Clb cyclin (Koch, Schleiffer et al. 1996). The Cdc28p/Clb5,6p complexes drive the completion of S-phase at the same time as the formation of Cdc28p/Clb1,2,3,4p complexes begin mitosis.

Figure 1.2

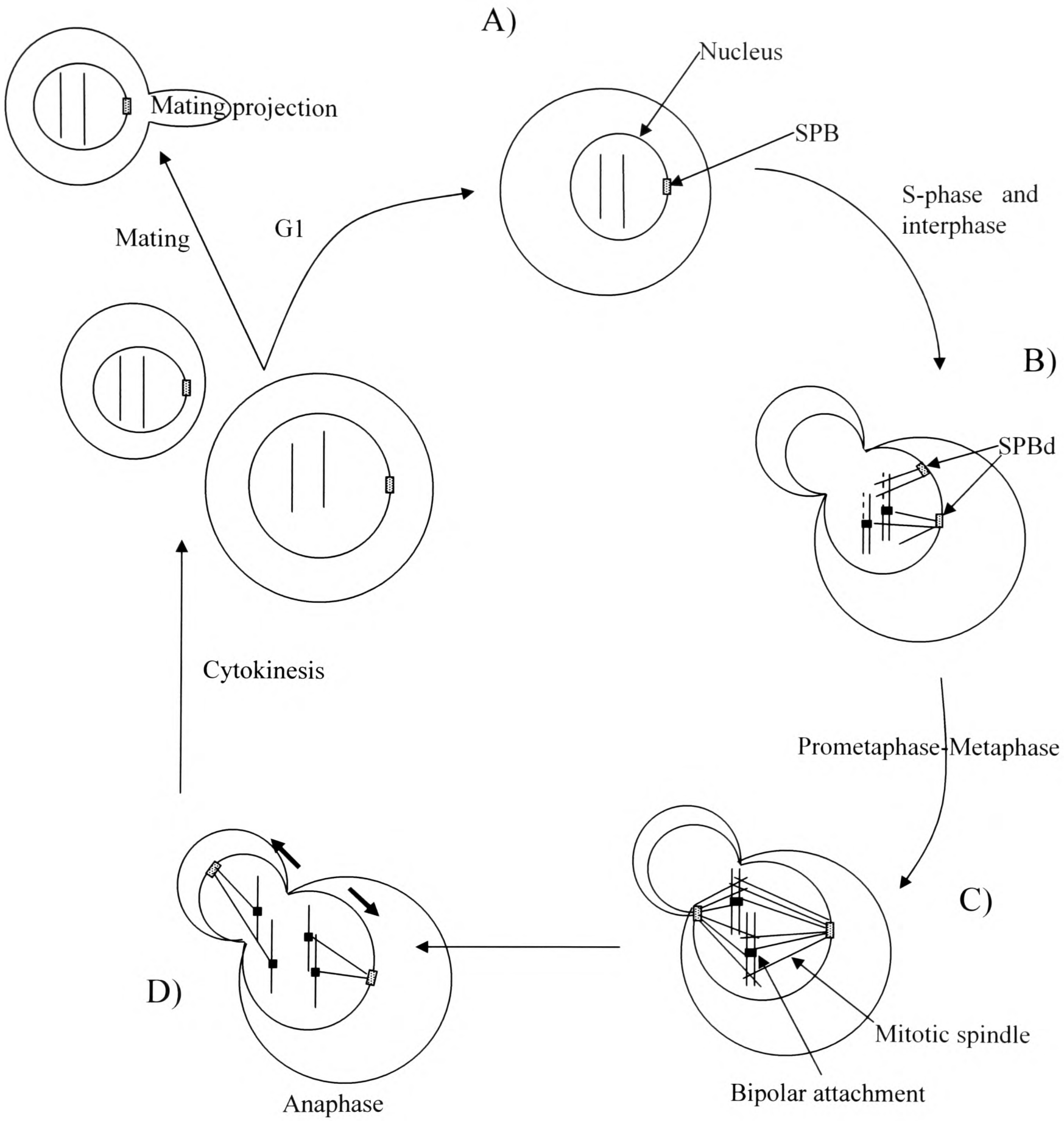


Figure 1.2 The cell cycle of *Saccharomyces cerevisiae*. The yeast cell cycle differs from the general eukaryotic cell cycle by firstly having a "closed" cycle, where the nucleus does not breakdown. A second difference is the appearance of a bud which must be negotiated by the dividing nucleus before the cell cycle begins again.

1.1.3 Mitosis

The pioneering work of microscopists sub-divided mitosis into five stages: prophase, prometaphase, metaphase, anaphase and telophase. Prophase is defined as the point at which the replicated chromosomes become dense and visible using a microscope and prometaphase begins when the centrosome (spindle pole body, SPB, in yeast) duplicates. Due to the small size of *S. cerevisiae* its chromosomes never become visible. In budding yeast prophase has already occurred during DNA replication with the concurrent duplication of the SPB (Figure 1.2 C). So it is difficult to identify prophase and prometaphase in budding yeast. Adding to these vagaries the continued presence of the nucleus, the breakdown of which signals prometaphase in higher eukaryotes, it is not a simple task to sub-divide budding yeast mitosis into these five phases.

These differences at first appear to be a good reason for rejecting this organism as a relevant species to study mitotic events. However, *S. cerevisiae* has a clear biochemical mitosis consisting of the sequential transition of the cyclins and the formation of mitotic structures such as the kinetochore which are universal in eukaryotes (Kitagawa and Hieter 2001). This, coupled to its genetic tractability and sequenced genome, making it a good organism on which to perform basic research into the cell cycle. Because of the conservation of many cell cycle components from yeast to mammals, the results from these experiments can often be translated to explain the more complex relationships between proteins in multicellular organisms, despite the differences between them.

The CDKs of organisms run growth and division in a cyclical manner. To ensure that each event - DNA replication, sister chromatid segregation, cytokinesis - occur in the correct order the cell has another level of control. Checkpoints monitor each step of the cell cycle and delay the onset of each stage until the previous stage has been completed. The mitotic checkpoint is the focus of this work (Section 1.3). This checkpoint monitors progression from metaphase to anaphase. This mitotic transition will now be discussed in detail.

In metaphase, even in *S. cerevisiae*, the sister chromatids are attached to a bipolar mitotic spindle. Upon anaphase initiation the sister chromatids are segregated

into the bud and mother cells. This event requires the attachment of the sister chromatids to the microtubules of the mitotic spindle. Microtubules are rod like polymers of the α–tubulin and β–tubulin proteins (Nogales 2001). They are nucleated, grown, from microtubule organising centres (MTOCs), which are termed SPBs in yeast, within the cell and used to transport vesicles and organelles around the cell (Tassin and Bornens 1999). In mitosis they are nucleated from the duplicated SPBs forming a mitotic spindle (Figure 1.2 D) (Knop, Pereira et al. 1999; McIntosh and O'Toole 1999). Some of the microtubules within this spindle connect with the kinetochores of the sister chromatids (Figure 1.2 D). Once kinetochores on a pair of sister chromatids have attached to opposite poles the chromatids are pulled towards opposite ends of the cell, or in the case of *S. cerevisiae*, pulled into the mother and daughter compartments of the cell. This process is incredibly complex and requires many events to occur correctly before it can happen with fidelity. The first structure required is the kinetochore.

1.1.4 The Kinetochore

The kinetochore is a protein complex formed on the centromere region of the chromosome (Figure 1.3). The centromere differs from organism to organism. The centromere in *Saccharomyces cerevisiae* consists of three distinct regions of the DNA called CDEI, CDEII and CDEIII (Clarke 1998). All three regions span only 125bp of DNA (Fitzgerald-Hayes, Clarke et al. 1982), this is only a fraction of the human regional centromere of 5Mbp (Craig, Earnshaw et al. 1999), but the basic function of either centromere must remain and in mitosis that is to form the base for a kinetochore. Centromere binding proteins attach to the centromere forming a kinetochore. These centromere proteins can be split into three functional groups; 1) DNA binding proteins found in the inner kinetochore, 2) kinetochore-microtubule interacting proteins found in the outer kinetochore, and 3) proteins at the interface between the previous two groups in the central kinetochore. Table 1.1 presents a list of the known kinetochore components of *S. cerevisiae* with their metazoan homologues, if known.

The DNA binding proteins form the base of the kinetochore and contain proteins with DNA binding activity such as Cse4p. Cse4p forms a complex with the

Figure 1.3

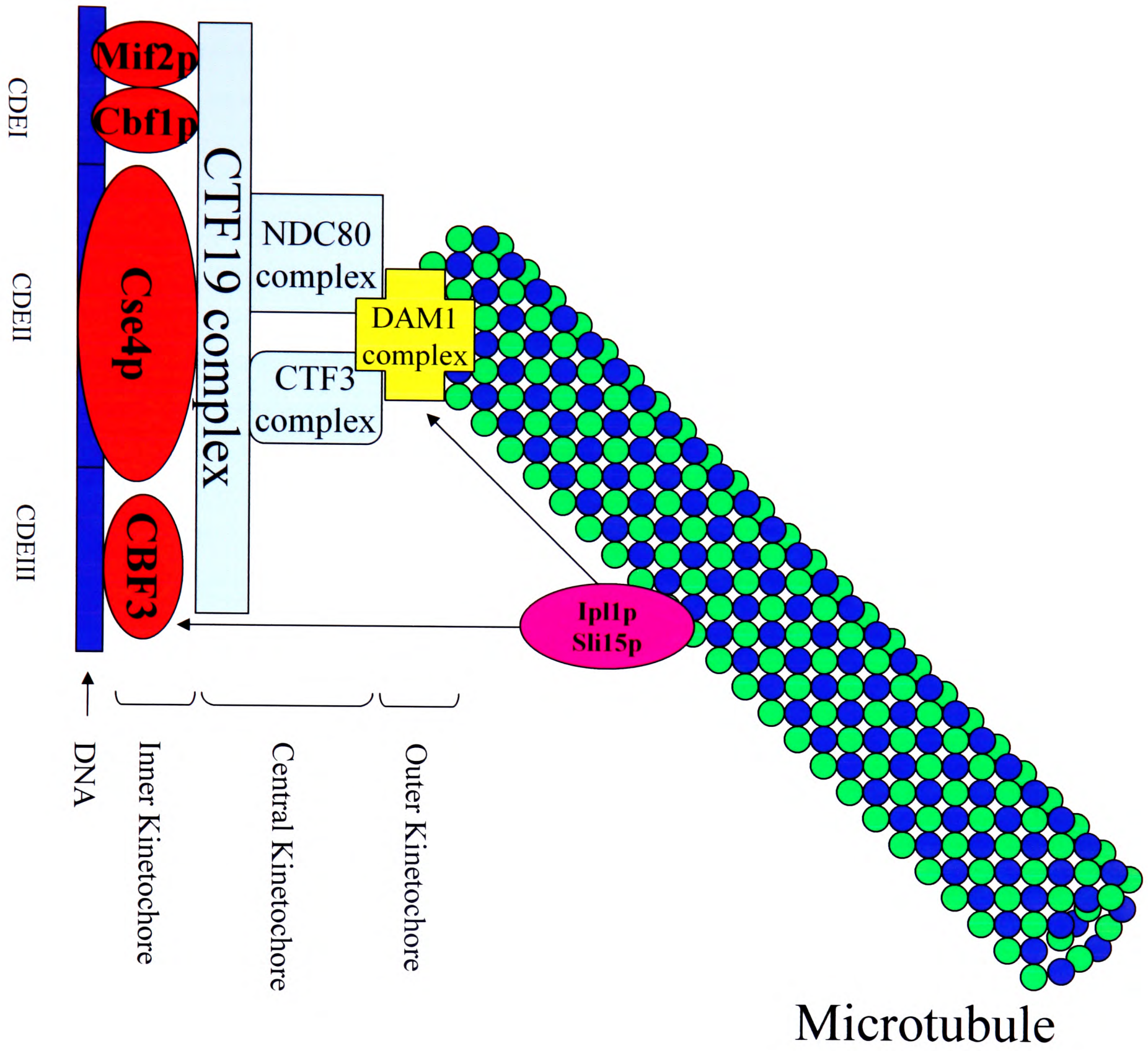


Figure 1.3 - The yeast kinetochore. The kinetochore is built on the foundations of the inner kinetochore components with the central components acting as a scaffold. The outer components interact with the microtubules of the spindle apparatus and the regulators of the kinetochore such as Ipl1p. A more detailed account of the yeast kinetochore is to be found in the text.

CDEII DNA of the centromere, with the CBF3 complex attached to the CDEIII DNA, and the Mif2p and Cbf1p proteins bound to CDEI. These DNA/protein complexes form the base of the yeast kinetochore and are represented in Figure 1.3.

Proteins in the outer kinetochore must attach to microtubules and pull the chromosomes to opposite poles when anaphase is initiated. As such some of the outer components contain a microtubule binding domain and a motor domain allowing them to attach to microtubules and actively move along them; for review see (Kull and Endow 2002). These microtubule motor proteins, the kinesins, together with dynein and the centromere protein, CENP-E, are known to mediate the kinetochore-microtubule interaction during mitosis in metazoans (Heald 2000). However, there is no yeast homologue for CENP-E and yeast dynein has a role in spindle positioning but not in kinetochore funtion (Heil-Chapdelaine, Oberle et al. 2000), so how does budding yeast perform the same role. Yeast mitotic kinesins have been found in the form of Cik1p, Vik1p, Cin8p, Kip3p and Kar3p and double mutants of *cin8Δ kip3Δ* and *kip3Δ kar3Δ* are indeed inviable (Miller, Heller et al. 1998). There is redundancy in the yeast kinesins as it is possible for mitosis to proceed with only Cin8p and either Kar3p or Kip3p (Cottingham, Gheber et al. 1999). Other non-motor microtubule associated proteins (MAPs) have been implicated in outer kinetochore function including the recently identified Dam1p complex (components listed in Table 1.1 and shown in Figure 1.3). The Dam1p complex has been shown to interact with the Ctf3 and Ndc80 complexes of the central kinetochore forming a link between the microtubules of the mitotic spindle and the centromere of the chromosomes (Cheeseman, Brew et al. 2001; Measday, Hailey et al. 2002).

In Figure 1.3 we see the structure of the yeast kinetochore with all the previously discussed components in context. The final part of introducing the kinetochore is to tackle the question of how it is regulated during mitosis. The spindle checkpoint is a key regulator of the kinetochore and its role will be discussed later Table 1.2 shows the regulators of the kinetochore. A relatively recent development in the understanding of kinetochore regulation has been the discovery of the protein kinase Ipl1p.

Ipl1p is a kinase with a number of distinct roles; bi-orientation of the kinetochores of sister chromatids and a role in the spindle checkpoint (this will be

discussed in section 1.2). Ipl1p together with its binding partner, and potentially its substrate specificity factor, Sli15p associates with kinetochores (Biggins and Murray 2001; Kang, Cheeseman et al. 2001). At the kinetochores Ipl1p can inhibit microtubule-kinetochore association *in vitro* by phosphorylating the CBF3 subunit Ndc10p (Biggins, Severin et al. 1999). Added to this Ipl1p can also phosphoyrlate Dam1p both *in vivo* and *in vitro* (Kang, Cheeseman et al. 2001). Thus it is clear that Ipl1p is not only associated with kinetochores, but can also phosphorylate kinetochore components and a consensus sequence for Ipl1p has been found in the Dam1p complex and the Ndc80p complex (Cheeseman, Anderson et al. 2002). *ipl1* and *sli15* mutants have high rates of chromosome missegregation and a high proportion of cells contain mono-orientated kinetochores (Biggins, Severin et al. 1999; Kim, Kang et al. 1999; He, Rines et al. 2001). This phenotype suggests that Ipl1p prevents chromosome missegregation perhaps through preventing or reversing mono-orientation of the kinetochores of sister chromatids. Tanaka *et al* have strengthened this case for a bi-orientation role for Ipl1 by showing that ipl1 mutants cannot detach kinetochores from the spindle pole during mitosis (Tanaka, Rachidi et al. 2002). Humans have three homologues of Ipl1p kinase (Aurora A, Aurora B and Aurora C) (Nigg 2001) and Aurora B has been shown to also be involved in the bi-orentation of sister kinetochores *in vivo* by using an inhibitor of the kinase (Hauf, Cole et al. 2003).

The mitotic checkpoint prevents the loss of chromosomes by ensuring the correct segregation of sister chromatids to the two daughter cells. To do this the mitotic checkpoint detects unattached kinetochores and kinetochores not under tension from the pulling forces of the spindle. The pulling forces of the spindle are strong enough to pull the centromeric regions of the sister chromatids apart what has been called the "breathing" of kinetochores (Goshima and Yanagida 2000; He, Asthana et al. 2000).

The complexity of the kinetochore and its structural and functional roles in mitosis make it an ideal focus for regulators of mitosis as has already been discussed with Ipl1p kinase. The components of the mitotic checkpoint, another regulator of mitosis, weave in and out of the kinetochore as they too perform their roles in mitosis as will now be discussed.

Table 1.1 - Components of the yeast kinetochore and their mammalian homologues

The Inner kinetochore

Yeast	Mammalian	Reference
CBF3 complex	Skp1	(Lechner and Carbon 1991)
Cbf1p	Cenp-B	(Niedenthal, Sen-Gupta et al. 1993)
Mif2p	Cenp-C	(Meluh and Koshland 1995)
Cse4p	Cenp-A	(Meluh, Yang et al. 1998)

The Central Kinetochore

Yeast	Mammalian (*S. pombe* underlined)	Reference
Ctf19 Complex	Mal2	(Ortiz, Stemmann et al. 1999)
Ctf3 Complex	Mis6	(Measday, Hailey et al. 2002)
Ndc80 Complex	Ndc80, Spc24, Him-10	(Janke, Ortiz et al. 2002)
Mtw1p	Mis12	(Goshima, Kiyomitsu et al. 2003)
Bir1p	Survivin	(Yoon and Carbon 1999)
Chl4p	SBP22H7.09c	(Roy, Poddar et al. 1997)
Mcm19p	None	(Ghosh, Poddar et al. 2001)

Table 1.1 - Components of the yeast kinetochore and their mammalian homologues (Continued)

The Outer Kinetochore

Yeast	Mammalian (***S. pombe*** bold, <u>Xenopus</u> underlined)	Reference
Dam1 Complex		(Cheeseman, Enquist-Newman et al. 2001)
Dam1p	SPAC589.8c	
Duo1p	SPBC3F12.08c	(Hofmann, Cheeseman et al. 1998)
Dad1p	SPAC16A10.05C	(Hofmann, Cheeseman et al. 1998)
Spc19	None	(Enquist-Newman, Cheeseman et al. 2001)
Spc34p	SPAC8C9.17C	
Ask1p	SPBC27.02c	(Janke, Ortiz et al. 2002)
Dad2p	SPAC1805.07C	(Janke, Ortiz et al. 2002)
Dad3p	None	(Janke, Ortiz et al. 2002)
Dad4p	None	(Janke, Ortiz et al. 2002)
Bik1p	Clip-170	(He, Rines et al. 2001)
Bim1p	EB1	(Tong, Evangelista et al. 2001)
Stu2p	<u>XMAP-215</u>	(Kosco, Pearson et al. 2001)
Cin8p	BimC knesin	(He, Rines et al. 2001)
Kip3p	XKCM1 kinesin	(Severin, Habermann et al. 2001)
Kar3p	NCD-like Kinesin	(Hildebrandt and Hoyt 2000)

Table 1.2 - Regulators of the yeast kinetochore

Component	Function	Reference
Mad1	Recruits Mad2 to the kinetochore	(Chen, Brady et al. 1999)
Mad2	Binds Mad1 and inhibits the APC	(Chen, Brady et al. 1999; Chen and Fang 2001)
Mad3/BubR1	Detects tension at kinetochore and inhibits the APC	(Skoufias, Andreassen et al. 2001; Tang, Bharadwaj et al. 2001)
Bub1	A Kinase which can phosphorylate Mad1 and Bub3.	(Roberts, Farr et al. 1994; Seeley, Wang et al. 1999)
Bub3	WD40 repeat protein. Binds to Bub1 and Mad3	(Hardwick, Johnston et al. 2000)
Mps1/TTK	Dual specificity kinase. Role in the Mitotic checkpoint	(Lauze, Stoelcker et al. 1995; Abrieu, Magnaghi-Jaulin et al. 2001)
Ipl1/Aurora	Role in biorientation and possible role in the mitotic checkpoint	(Biggins and Murray 2001; Tanaka, Rachidi et al. 2002)
CENP-E	Binds to BubR1. No clear yeast homologue	(Chan, Schaar et al. 1998; Yao, Abrieu et al. 2000)

1.2 Cell cycle checkpoints

All metazoans originate from a single cell containing all the information needed to produce the adult organism. This involves a staggering number of cell cycles with all the complexity that each cell cycle contains it is amazing that genetic diseases such as cancer are so rare – cancer affects 1 in 3 people throughout a lifetime (Information and Statistics Division (ISD), NHS, George Street, Edinburgh, Scotland). Like a CD player reading a compact disc or the security services at an airport, the cell has built in safeguards checking for errors in the system. These safeguards are termed checkpoints.

The arrests caused by the checkpoints allow the following: (I) Cellular damage to be repaired, including the malfunction of a cell cycle process such as mitosis, (II) The dissipation of an exogenous cellular stress signal, and (III) the availability of essential growth factors, hormones, or nutrients. Once the problem has been solved the checkpoint arrest ends and the cell proceeds onto the next stage. A checkpoint can be defined as a mechanism which does not partake in the functional progression of the cell cycle but instead is external to the cell cycle and contains a sensor to detect errors, a transducer to transmit the error signal and an effector to right the error (Nyberg, Michelson et al. 2002).

The first checkpoint to be found was the DNA damage checkpoint of budding yeast. Figure 1.4 shows the checkpoints of budding yeast. The existence of checkpoints had been suspected but it was Weinert and Hartwell who devised a screen to find mutant yeast which were unable to arrest and repair their DNA in response to X-ray irradiation (Weinert and Hartwell 1988). Using an assay to count the divisions of individual cells (a microcolony assay), a mutant in the *RAD9* gene was identified as unable to arrest cells irradiated with X-rays. The Rad9p was found to be a sensor in the DNA damage checkpoint (Volkmer and Karnitz 1999) and is found associated with damaged DNA (Lindsey-Boltz, Bermudez et al. 2001). The Rad9p complex, called the 9-1-1 complex, along with other factors lead to the activation of signal transducers such as the Chk1p protein which ultimately results in the arrest of cell cycle progression and the activation of DNA repair mechanisms (Walworth and Bernards 1996). DNA replication must also be monitored as a stalled replication fork can lead to

Figure 1.4

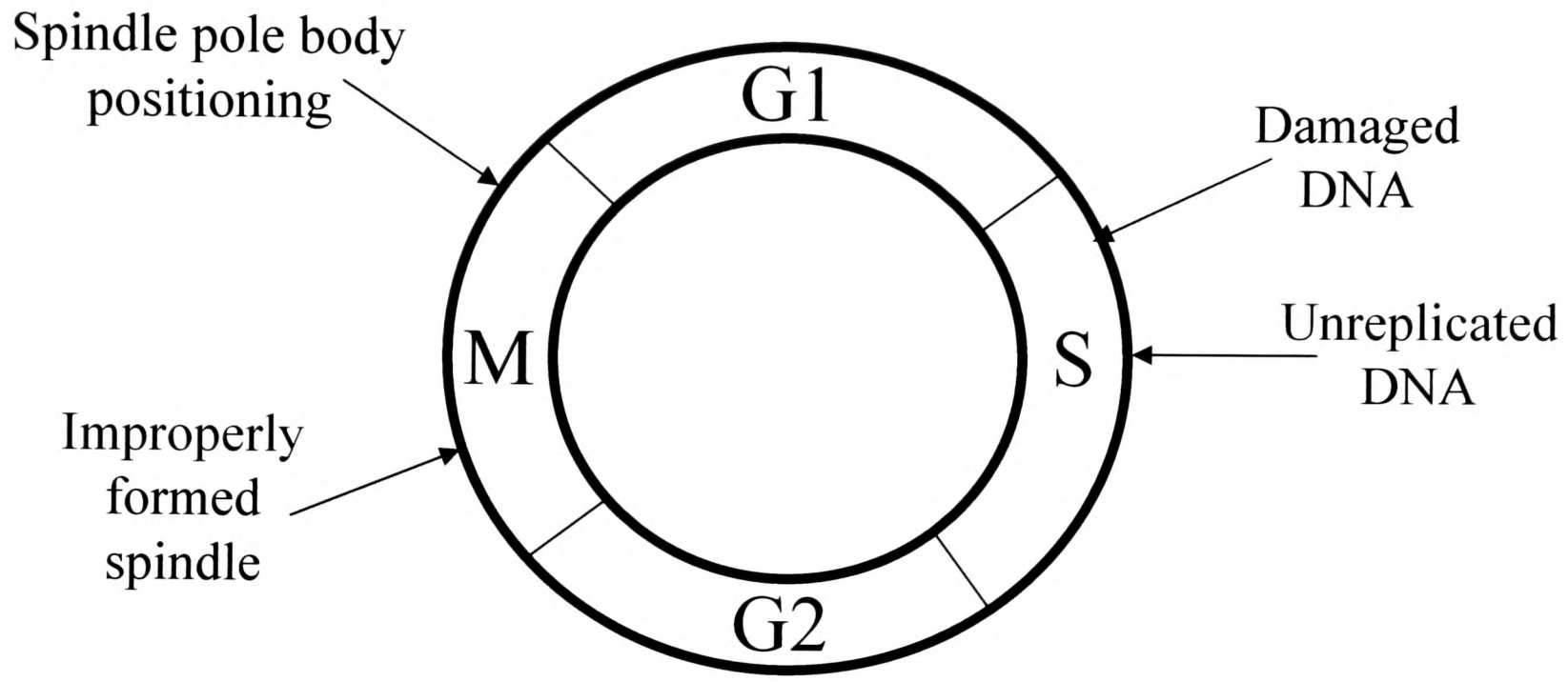


Figure 1.4 - The checkpoints of the cell cycle. The cell responds to these purturbations by arresting the cell cycle, allowing time to correct the problem. For reviews on each checkpoint see text.

chromosome rearrangements and then to genome instability. The DNA replication checkpoint in yeast requires the Mec1p and Rad53p proteins to block DNA replication and to initiate a response to the error in replication (Weinert, Kiser et al. 1994; Santocanale and Diffley 1998; de Klein, Muijtjens et al. 2000). The third checkpoint found in budding yeast responds to the incorrect positioning of the bipolar spindle in the bud neck (Figure 1.4). This checkpoint ensures the completion of mitosis does not occur until the nucleus is positioned at the neck of the bud. The *BUB2* gene originally discovered in the screen by Hoyt *et al* for checkpoint components is involved in this checkpoint (Hoyt, Totis et al. 1991; Fraschini, Formenti et al. 1999). For a review of the Bub2p dependent spindle position checkpoint see (Schuyler and Pellman 2001). The forth, and so far the final, checkpoint in budding yeast monitors the mitotic spindle apparatus and will be discussed in more detail in the following sections.

1.2.1 The Mitotic Checkpoint

The work by Weinert and Harwell on the DNA damage checkpoint spurred other workers to look for checkpoints elsewhere in the cell cycle. And in 1991 the Murray and Hoyt laboratories discovered a feedback mechanism in mitosis which responded to lesions in the spindle machinery, they called this mechanism the spindle checkpoint. In modern times it is referred to as the mitotic checkpoint.

Li and Murray discovered the *MAD* (mitotic arrest deficient (Li and Murray 1991)) genes and Hoyt *et al* discovered the *BUB* (budding uninhibited by benomyl (Hoyt, Totis et al. 1991)) genes. Mutants in *MAD1,2,3* (Li and Murray 1991) or *BUB1,2,3* (Hoyt, Totis et al. 1991) make yeast cells sensitive to microtubule depolymerising drugs such as nocodazole and benomyl. By monitoring the growth of these cells using microcolony assays (Figure 1.5), which measure the rate of division of a single cell, and by monitoring the time at which these cells segregate their chromatids in the presence of nocodazole, it was discovered that these genes are involved in delaying the onset of anaphase in response to errors in spindle construction (Amon

Figure 1.5

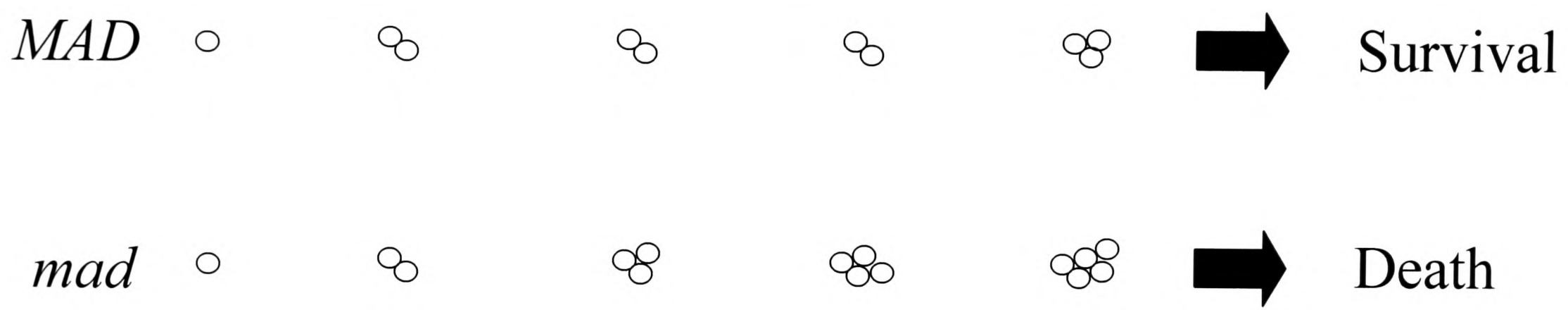


Figure 1.5 - The yeast microcolony assay. *MAD*, wild type yeast. *mad*, checkpoint mutant. *tub1*, tubulin mutant. The assay was performed in the presence of benomyl which depolymerises microtubules. The *MAD* cells divide slowly as the functional mitotic checkpoint is delaying cell cycle progression in response to spindle perturbation by benomyl. The *tub1* cells cannot divide and die, as they contain a structural mutation which also makes them benomyl sensitive. Finally, the *mad* cells continue to divide in spite of spindle perturbations resulting in cell death due to chromosome loss.

1999). The Hoyt group analysed a population of mutated cells by growing them in the presence of a high concentration of benomyl (70ug/ml) and replica plating them onto nutrient media lacking benomyl. By assaying the growth of these replica-plated colonies, sensitivity to benomyl was established. It seems strange that in two separate screens for the same type of mutant no overlap in the discoveries were made. However, in a subsequent screen by the Murray group *bub* mutants were found, proving that the initial screens by both groups were not saturating (K. Hardwick; per. comm).

None of these genes are essential in yeast although in mouse cells Mad2 knockout are lethal at the embryonic stage of development (Dobles, Liberal et al. 2000). The non-essential nature of the checkpoint components was expected in budding yeast since under ideal growth conditions the cell cycle proceeds with no perturbations and does not require the presence of a checkpoint. In early 1996 Weiss and Winey published a paper showing that mutations in an essential gene, *MPS1* were also checkpoint defective (Weiss and Winey 1996). The authors of this paper suggests that the *MPS1* protein, by having a dual role in SPB duplication, is in a good functional position to signal to the checkpoint machinery that a defect has arisen in an essential part of mitosis.

In their second screen the Murray group also found another mutant which was defective in the spindle checkpoint, *cdc55* (Minshull, Straight et al. 1996); (Wang and Burke 1997). *CDC55* encodes a regulatory subunit of yeast PP2A phosphatase, a *cdc55Δ* has a similar phenotype to the *mad/bub* mutants but it differs in that although CDK kinase activity drops as the cells exit mitosis, cyclin levels remain high. This led both the Minshull and Wang groups to suggest that Cdc55p is not a member of the checkpoint machinery but instead is part of an adaptation pathway for cells to escape the arrest and it does this by inhibiting Cdc28p activity through dephosphorylation.

What is the substrate(s) of Mps1p kinase? The Mps1p kinase activity peaks during G1/S when the SPB is being duplicated, and during mitosis in the presence of nocodazole (M. Winey; per comm.). A galactose induced Mps1p was over-expressed in cells and caused an arrest at the metaphase to anaphase transition with elevated levels of Clb2p and CDK kinase activity. This over-expression of *MPS1* was able to

activate the spindle checkpoint in the absence of microtubule depolymerising drugs and led to a dramatic hyperphosphorylation of Mad1p (Hardwick, Weiss et al. 1996). Mad1p is also phosphorylated in the presence of nocodazole. Indeed Mad1p is a substrate of the Mps1p kinase *in vitro*, although the significance of this phosphorylation is uncertain as the phosphorylation sites on Mad1p have yet to be mapped and thus altered. The phosphorylation state of Mad1p in nocodazole arrested cells was used to define an order of the Mad and Bub proteins in the checkpoint pathway. Mutants where Mad1p does not get phosphorylated in the presence of nocodazole were placed upstream of Mad1p in the checkpoint pathway, mutants not required for this phosphorylation were placed downstream (Figure 1.6).

This work gave a general view of where these genes acted in the spindle checkpoint. It is tempting to postulate roles for the proteins from this "pathway", placing Mps1, Bub1p and Bub3p at the initiation of the arrest signal, with possible roles in the detection machinery. But evidence suggests that the checkpoint is more complex than suggested in the diagram; for instance, Bub3p interacts with Mad3p *in vitro* (K. Hardwick; per. comm.) yet they are at opposite ends of the pathway suggesting that they have multiple roles. Mad2p is also placed at the top of the pathway suggesting a sensor/transducer role yet the bulk of evidence to date places points to an effector role for Mad2p (Luo, Fang et al. 2000; Chen and Fang 2001).

1.2.2 What does the checkpoint detect?

It is clear from the initial screens using benomyl that it detects spindle depolymerisation, but yeast or mammalian cells bathed in rich growth media are unlikely to encounter these conditions, so are there more subtle problems which the checkpoint detects? Work done in mammalian cells has shown that the checkpoint can detect a single unattached kinetochore and arrest the cell cycle until it is attached to the bipolar spindle (Rieder, Schultz et al. 1994). When the kinetochore was demolished using a laser, the cell entered anaphase after a short delay, so a kinetochore is required

Figure 1.6

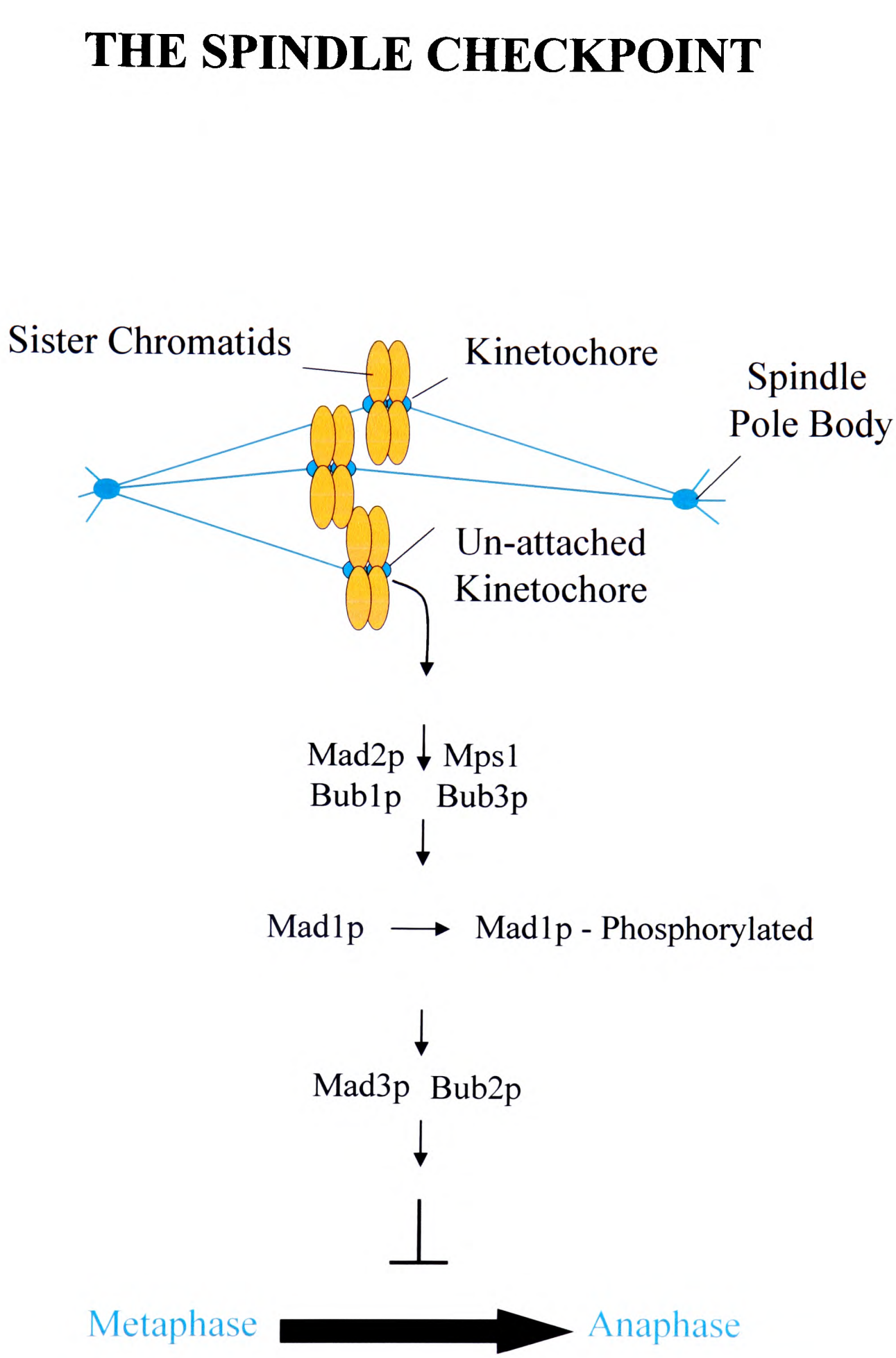


Figure 1.6 - Epistatic relationships within the mitotic checkpoint. It was hypothesised that unattached kinetochore or an absence of tension would trigger the checkpoint. A signal would then be sent through the above pathway (adapted from Hardwick *et al*, Science, 1996: 273, pp953-6). This hypothesis suggests a simple signal transduction pathway where Mad2p, Bub1p, Bub3p and Mps1p act before Mad1p phosphorylation and would therefore be involved in signal transduction. Mad3p and Bub2p would then act after the phosphorylation of Mad1p and would be involved in the final inhibition of anaphase.

for segregating the chromatids and to signal the checkpoint machinery; perhaps a signalling apparatus resides on the kinetochore?

The checkpoint not only responds to a single unattached kinetochore but also to multiple artificial chromosomes. This was shown by Wells and Murray when they used pedigree analysis to track minichromosomes in *S. cerevisiae* (Wells and Murray 1996). They showed that the checkpoint was activated not only by poorly segregating short linear chromosomes but also by multiple copies of circular chromosomes. This arrest was abolished in *mad/bub* mutants exhibiting the involvement of the checkpoint in this arrest. The high copy number of the circular minichromosomes may be titrating out a limited number of kinetochore proteins or, more simply, the increased number of chromosomes leads to a greater chance of one being unattached to the spindle. The linear chromosomes demand a more complex explanation, as they have centromeres which work on larger chromosomes, however, their small size may make them vulnerable to the "polar wind". Microtubules push on chromosome arms and repel them from the pole, projecting them towards the centre of the spindle (Rieder and Salmon 1998). As the small chromosomes have short arms this force does not have an effect, and the chromosomes are pulled towards a pole, reducing the chance of being bipolar attached. However, there is no evidence for the existence of a polar wind in yeast. Whatever the reasons for this it is clear from the above experiments that an unattached kinetochore can arrest mitosis via the spindle checkpoint. It is not fully understood how this happens but experiments in insect germ line cells suggest that tension is being sensed.

When chromatids attach to the spindle they are pulled on by the attached microtubule(s). If the chromosome is attached to both poles it will be under tension via the opposite pulling forces of either pole. The strength of this tension is visible in yeast cells which have a centromere marked with the green fluorescent protein, GFP. In these cells the green signal from the centromeres of paired chromatids can be seen being pulled apart, this phenomenon has been called the "breathing" of the centromeres (Goshima and Yanagida 2000; He, Asthana et al. 2000). A monopolar attached chromosome will not be under tension (McIntosh and Hering 1991). Nicklas investigated the effect of tension on male mantid spermatocytes (Li and Nicklas 1997).

These cells have two X chromosomes and one Y chromosomes, during meiosis I they sometimes form a trivalent; two X's attached to one Y, with all attached to the spindle. This structure is unstable so there are many cells with a monopolar attached X chromosome. These cells are arrested in late metaphase, however, the arrest is abolished when the monopolar chromosome is tugged by a micromanipulating needle.

If one follows the phosphorylation state of an unknown kinetochore associated protein(s) using the 3F3/2 antibody when these manipulations are occurring, the unattached kinetochore stains brightly (Nicklas, Ward et al. 1995) (Gorbsky and Ricketts 1993). This staining is reduced by the addition of tension on the chromosome, again by the needle. Such 3F3/2 epitopes are not restricted to the kinetochore, they also appear on the centrosome. So, a protein(s) at the kinetochores/centrosomes becomes phosphorylated when the chromatids are incorrectly attached to the spindle, what is the identity of this protein(s)?

Mad1p in yeast (Jin, Spencer et al. 1998), Mad2 in vertebrates (Gorbsky, Chen et al. 1998) hBub1 and hBubR1 (Taylor and McKeon 1997) and hBub3 (Taylor, Ha et al. 1998) antibodies stain both the kinetochores of mammalian cells but only Mad1p, Bub1p and hBubR1 have been shown to be phosphorylated (Hardwick and Murray 1995; Taylor, Hussein et al. 2001). Mad1p is a 90kD nuclear phosphoprotein with a central coiled-coil domain (Hardwick and Murray 1995). The coiled-coil domain may be stretched when not under tension, exposing phosphorylation sites, but this is only a theory and has yet to be tested. Gorbsky *et al* localised mammalian Mad2 to the kinetochore of Ptk1 cells and primary cultures (foreskin keratinocytes) (Gorbsky, Chen et al. 1998). Mad2p binds tightly to Mad1p in yeast so it is likely that the same is true for mammalian systems, but an equally important result was also yielded from this paper. When anti-Mad2 antibody was injected, the cells exited mitosis significantly earlier than controls. This result was also obtained for hBub1 (Taylor and McKeon 1997). Taylor and McKeon localised the Bub1 protein kinase to the kinetochore of prophase and prometaphase cells, and showed that transiently transfecting cells with a fragment of hBub1 containing the kinetochore localisation region (later found to be the Bub3 binding domain) induced exit from mitosis approximately 20 minutes before control cells. There is only preliminary data to support a timing function for checkpoint

proteins in yeast. *bub1Δ* and *bub3Δ* cells are very sick in the absence of benomyl, perhaps the timing of anaphase in the cells is disrupted (Taylor and McKeon 1997). These experiments implicate the spindle checkpoint in the timing of anaphase, not only preventing anaphase if the spindle is disrupted with drugs but preventing it in a normal mitosis until the sister chromatids are aligned on the metaphase plate and bipolar attached. The localisation of these checkpoint components to the kinetochore of metaphase cells is an important addition to the evidence that has been gleaned from experiments in yeast.

1.2.3 How does the spindle checkpoint prevent anaphase?

When the spindle checkpoint arrests cells at the metaphase to anaphase transition, CDK activity remains high and Clb2p (the major budding yeast mitotic cyclin) levels also remain high (Murray, Solomon et al. 1989). Normally Clb2p is degraded late in anaphase, eliminating Clb2/CDK activity. A number of important cell cycle regulators are degraded at various stages of the cell cycle; Sic1p at the G1/S transition, allowing S-phase to proceed (Nishizawa, Kawasumi et al. 1998); Pds1p at metaphase (Shirayama, Toth et al. 1999); Clb2p and Ase1p during anaphase in *S. cerevisiae* (Hoyt, Macke et al. 1997; Juang, Huang et al. 1997). To understand how the checkpoint inhibits anaphase it is necessary to understand this mechanism of degradation.

All of these proteins are marked for proteolysis by the addition of a chain of the ubiquitin protein. Ubiquitin is a small highly conserved protein found, as its name suggests, ubiquitously. The ubiquitin chain leads to the degradation of the attached protein by the 26S proteosome; a multimeric complex which degrades proteins throughout the cell (Coux 2002). How this ubiquitin chain becomes attached differs for each protein (Pickart 1997).

The process of ubiquitination is outlined in figure 1.7. A ubiquitin activating enzyme, or E1, first activates ubiquitin by forming a thioester bond with it. A ubiquitin conjugating enzyme (E2) then binds the activated ubiquitin. This process continues, building a chain of ubiquitin on the E2. Next, the ubiquitin chain is transferred from the

Figure 1.7

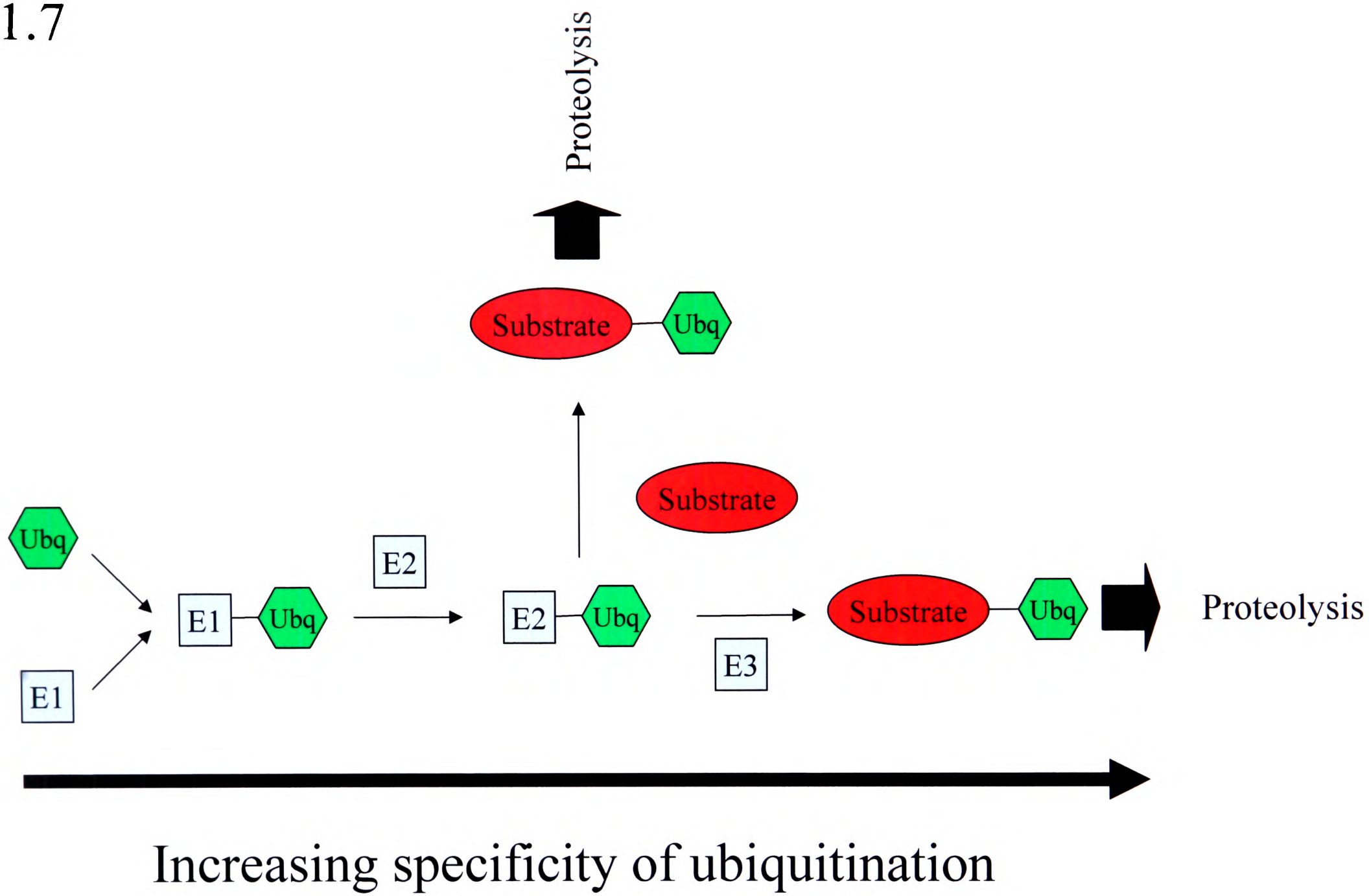


Figure 1.7 - The road to proteolysis by the 26S proteosome. The ubiquitin molecule (green hexagon) is first activated by the E1 enzyme. Once activated the E2 enzyme can transfer the ubiquitin onto the substrate directly. In many cases a second conjugating enzyme, the E3, can increase the specificity of the system by targeting the ubiquitin onto the substrate. In this way proteins can be selected for proteolysis with a high degree of specificity.

E2 onto the substrate. In some cases this transfer happens directly; the E2 transfers the ubiquitin chain onto the target protein. In this way the E2 confers some specificity to the ubiquitination process; there are 13 E2s in the *S. cerevisiae* genome (Townsley and Ruderman 1998). In many cases, however, a third enzyme, the E3, transfers the ubiquitin chain from the E2 to the substrate (Figure 1.7). In the case of Sic1p this E3 is called the Skp1-Cdc53-Fbox protein complex (SCF) (Jackson and Eldridge 2002). The SCF consists of three proteins, Skp1p, Cdc53p and an F-box protein. Skp1p and Cdc53p form the central part of the complex, the F-box protein varies for each group of substrates, conferring another level of specificity. Cdc4p acts as the F-box protein during the ubiquitination of Sic1p (Verma, Feldman et al. 1997).

In order to proceed through anaphase the cell must degrade at least three proteins; Pds1p, Clb2p and Ase1p. In these cases an E3 is required to add the ubiquitin to the these target molecules. The E3 responsible for their ubiquitination is known as the anaphase promoting complex (APC) or cyclosome (Peters 2002). The APC consists of at least 12 subunits in *S. cerevisiae*, 10 of which are thought to be conserved in all eukaryotes; Apc1p, Cdc16p, Cdc23p, Cdc26p, Cdc27p, Apc10p Apc2p, Apc4p, Apc5p and Apc11p. All the known substrates of the APC have one or more copies of a destruction box (D-box) sequence (Burton and Solomon 2001). This sequence targets proteins to the APC for ubiquitination leading to their subsequent degradation by the 26S proteosome; if the D-box is deleted from the gene of say *PDS1*, the protein will not be degraded (Shirayama, Toth et al. 1999).

Both Pds1p and Clb2p have D-box sequences as does Ase1p. Ase1p and Clb2p are degraded during anaphase allowing the disassembly of the mitotic spindle and exit from mitosis. Pds1p is the only protein whose degradation is required for sister chromatid separation and the subsequent initiation of anaphase (Lim, Goh et al. 1998). As the checkpoint must inhibit anaphase it is clear that it must prevent the degradation of Pds1p.

Pds1p prevents anaphase by inhibiting the separation of the sister chromatids. When the DNA is duplicated it is essential to form a link between the sister chromatids. If this link was not formed the sister chromatids would drift apart in the cell and bipolar segregration would be, at least, problematic. The link is formed by the addition of cohesin molecules at the interface of the sister chromatids by components of the DNA replication machinery (Skibbens, Corson et al. 1999; Mayer, Gygi et al.

2001). Cohesin is composed of four molecules; Scc1p, Scc3p, Smc1p and Smc3p. The coiled-coil Smc1p and Smc3p are thought to form a ring dimer with a head domain and a hinge domain opposing each other in the ring (Figure 1.8) (Uhlmann 2003). The Scc1p associates with the head domain of each Smc protein with the ring surrounding the DNA strands of both sister chromatids.

The proteolysis of Scc1p by Esp1 is the final step in anaphase initiation. Once this has happening the sister chromatids are no longer bound together and the pulling forces of the opposing spindle poles can pull them towards each pole. The proteolysis of Pds1p is essential to allow this as Pds1p (also called securin) associates with Esp1p throughout mitosis and prevents its activity towards Scc1p.

So the checkpoint must inhibit Pds1p proteolysis to prevent premature anaphase onset. The mitotic checkpoint components do not associate with Pds1p directly to prevent its proteolysis but instead they combine to prevent its proteolysis by inhibiting APC activity towards Pds1p.

The E2 of the ubiquitination machinery confers specificity for certain substrates and the E3 (APC in mitosis) supplies additional specificity but as Pds1p, Clb2p and Ase1p are degraded by the APC at different times, other proteins must alter the specificity of the APC. Two such proteins are Cdc20p and Hct1p. Both proteins contain WD40 repeats in their primary structure, these repeats have been implicated in protein-protein interactions and form a "propeller" motif in the tertiary structure (Renault, Nassar et al. 1998). Current research suggests that Cdc20p targets the APC towards Pds1p(Visintin, Prinz et al. 1997). Hct1p targets the APC towards Clb2p and Ase1p (Schwab, Lutum et al. 1997) (Figure 1.9). If Cdc20p was inhibited in some way, the APC would be unable to ubiquitinate Pds1p and anaphase would be inhibited.

Is there any evidence that Cdc20p is inhibited by the spindle checkpoint? Several lines of evidence do suggest this. In budding yeast Mad1p, Mad2p and Mad3p coimmunoprecipitated with Cdc20 (Hwang, Lau et al. 1998). Similar evidence for Mad2 association with Cdc20 has been found in the fission yeast, *Schizosacharomyces pombe* (Kim, Lin et al. 1998) and mammalian cells (Kallio, Weinstein et al. 1998). This evidence places the checkpoint components with Cdc20p but the most convincing evidence came from the analysis of mutations in the *CDC20* gene of *S. cerevisiae* and

Figure 1.8

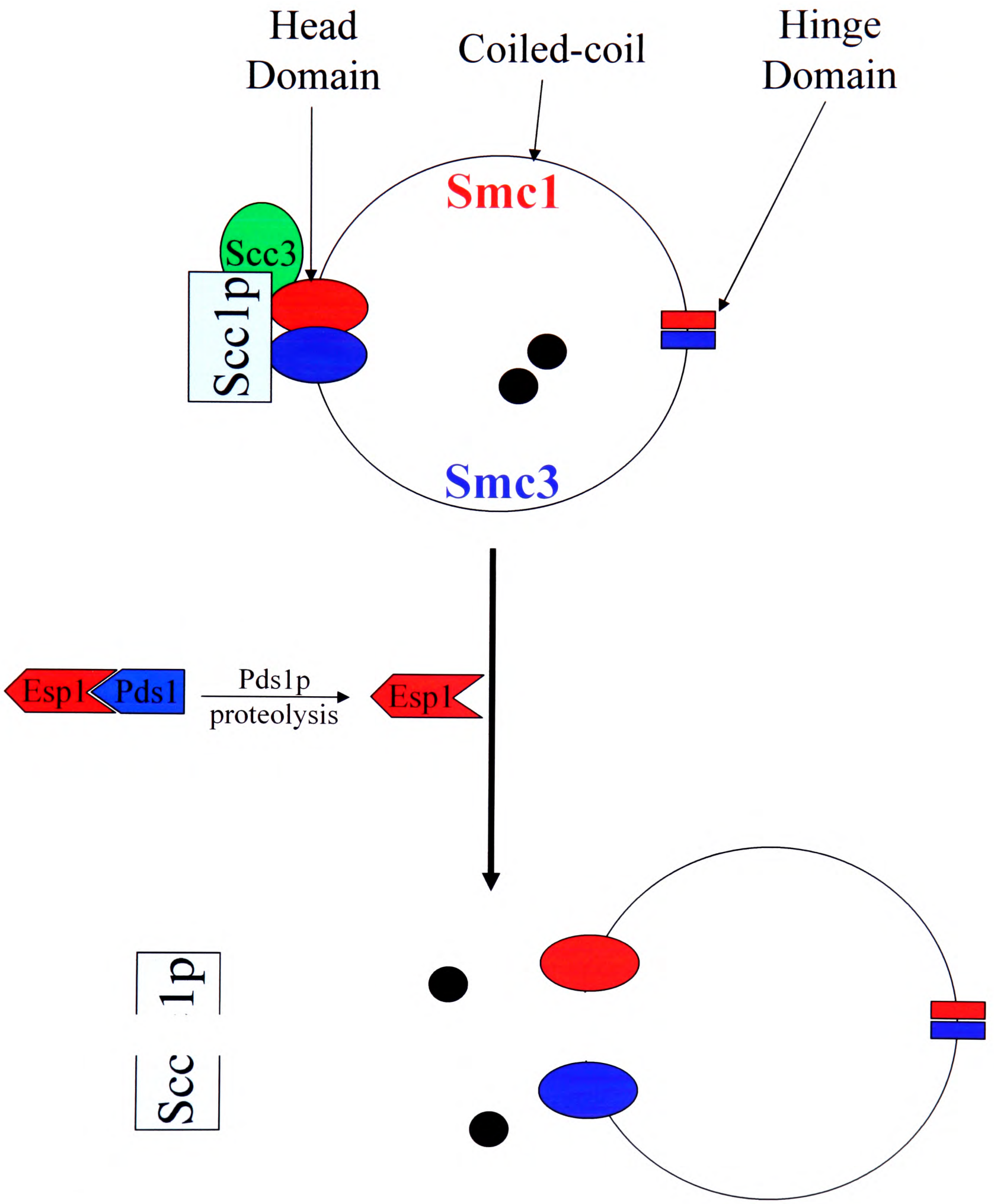


Figure 1.8 - The ring of cohesin and its destruction. The sister chromatid DNA (black circles) is held inside the cohesin ring until the proteolysis of Scc1p by freed Esp1p. It is hypothesised that when this happens the ring opens from its hinge domain and allows the sister chromatids to be released. Adapted from Uhlmann, 2003.

Figure 1.9

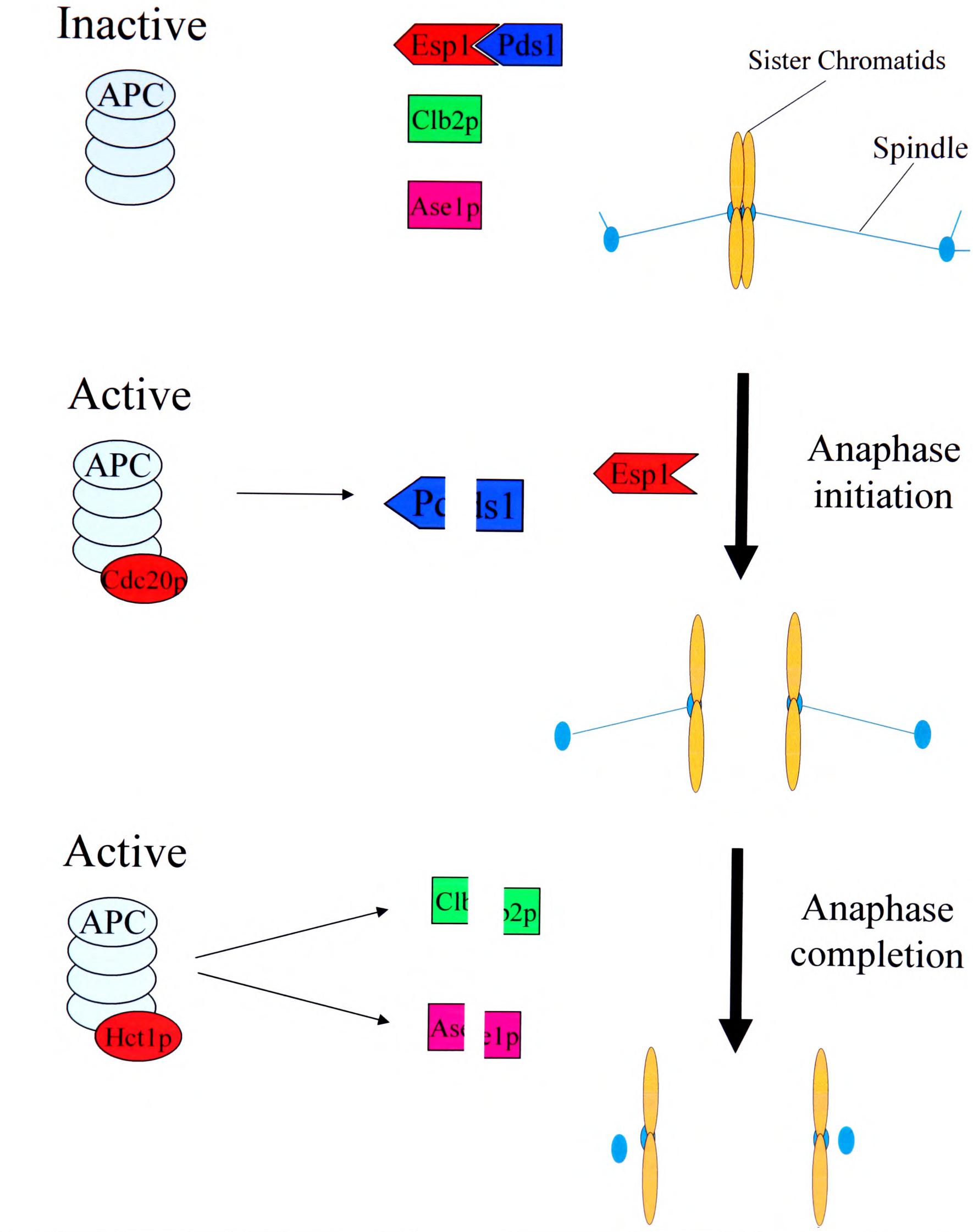


Figure 1.9 - The activity of the APC/Cyclosome through mitosis. Once APC-Cdc20p is activated at the initiation of anaphase it adds chains of ubiquitin to Pds1p, triggering its destruction and allowing anaphase initiation. Subsequently, during anaphase Hct1p can target the APC to add chains of ubiquitin to Clb2p and Ase1p allowing their destruction by the proteosome leading to the ultimate completion of anaphase.

its *Schizzosacharomyces pombe* homologue, Slp1. Hwang *et al* discovered point mutations in *CDC20* which prevented the association of Cdc20p with the checkpoint components and found these mutants to be checkpoint defective (Hwang, Lau et al. 1998). This group used the over-expression of *GAL-MPS1* to arrest the cells in mitosis. They then isolated mutants of *CDC20* that could no longer arrest under these conditions. The isolated mutants contained point mutations in the N-terminal region of Cdc20p and could no longer bind to the checkpoint components. This strongly suggested that Cdc20p was the target of the mitotic checkpoint. Kim *et al* were able to arrest cells in mitosis by over-expressing Mad2 and screened for mutations in $slp1^+$ that would overcome this arrest. These $slp1^+$ mutants could not bind to Mad2 in *S. pombe* (Kim, Lin et al. 1998). These two papers were key in identifying Cdc20p as the key target of the spindle checkpoint.

As Cdc20p is a sub-stoichiometric part of the APC during mitosis, it is possible that Cdc20p localises checkpoint components to the APC, allowing its complete inhibition. Indeed p55CDC, the human homologue of Cdc20p, mediates association between the APC and Mad2p in mammalian cells (Kallio, Weinstein et al. 1998). So by inhibiting Cdc20p function and thereby proteolysis, the checkpoint inhibits anaphase.

1.2.4 How is the signal relayed from detector to effector?

A signal is produced from an unattached kinetochore or a malformed spindle (via Mps1p?) which inhibits proteolysis via the APC. How is this signal transduced from the sensor (possibly a phosphorylated checkpoint component on the kinetochore, Mad1p?) to the effector (Mad/Cdc20p)? The signal cascade probably involves the two kinases in the checkpoint; Mps1p and Bub1p. These proteins are dependent on each other as *BUB1* is required for an arrest in cells over-expressing Mps1p and Mps1p is required in cells containing a dominant allele of *BUB1* (*BUB1-5*) which activates the checkpoint in the absence of spindle disruption (Farr and Hoyt 1998). A difference in their function is implied by an experiment in budding yeast which showed that Mad1p

phosphorylation was not needed for an arrest in *BUB1-5* cells (Farr and Hoyt 1998). Mad1p is always phosphorylated in cells over-expressing *MPS1*. This suggests that they are indeed dependent on each other and their mechanism of activating the checkpoint involves the modification of Mad1p.

Rieder *et al* addressed the question of a signal cascade by monitoring fused cells with two functional spindles (Rieder, Khodjakov et al. 1997). He noted that a single unattached kinetochore arrested its own spindle but had no effect on the adjacent spindle 20μm away, illustrating beautifully that the signal is not far reaching.

1.2.5 The spindle checkpoint and disease

When bathed in the luxury of laboratory conditions the humble yeast cell, or any single cell, is not exposed to the woes of the world. When cells find themselves in the real world of carcinogens, mechanical stress, starvation and poisons, the cell cycle checkpoints become more important. The spindle checkpoint in yeast is expendable as can be shown by the non-essential nature of its components, in metazoans it is absolutely essential for normal growth and division.

Mutations in the mitotic checkpoint in the worm *Caenorhabditis elegans* lead to sterility and a short life span in the adult (Kitagawa and Rose 1999). The situation is worse in the fruit fly, *Drosophilia melanogastor*, as disruption of *Bub1* causes lethality at the larva-pupa transition (Basu, Bousbaa et al. 1999). The absence of *Mad2* or *Bub3* from mice also causes lethality at the early embryonic stage, confirming the importance of the mitotic checkpoint to the development of metazoans (Dobles, Liberal et al. 2000; Kalitsis, Earle et al. 2000). Deletion of one allele of *Mad2* in mice (forming a $Mad2^{+/-}$ mouse) leads to an increase in lung cancer in the adult suggesting that a malfunctioning mitotic checkpoint may be implicated in some cancers (Michel, Liberal et al. 2001). Indeed mutations in *Bub1* and *BubR1* co-operate with *BRCA2* deficiency to exacerbate breast cancer (Lee, Trainer et al. 1999) and Mad2 expression levels have been linked to ovarian cancer (Wang, Jin et al. 2002). Dominant alleles of *Bub1* may be responsible for the chromosomal instability phenotype of some colorectal cancers

although there is some doubt in this as the checkpoint in these cells was functional (Cahill, Lengauer et al. 1998; Tighe, Johnson et al. 2001).

The consequences of disrupting the mitotic checkpoint is not only found in somatic tissue but as the *C. elegans* sterile phenotype suggests, the germ line of an organism can also be affected. This is supported by the recent implication of the checkpoint components in the meiosis of yeast (Shonn, McCarroll et al. 2000; Bernard, Maure et al. 2001). Errors in chromosome segregation in the germ line can lead to missing or extra chromosomes in the offspring. This may lead to a miscarriage of the foetus or the advent of disease in the child such as Patau syndrome, Edwards syndrome, Down syndrome and Klinefelter syndrome which result from an extra copy of chromosome 13, chromosome 18, chromosome 21 and the X chromosome, respectively (Sluder and McCollum 2000).

This evidence creates another avenue for exploring the causes and progression of disease. It is likely that use of drugs to affect components of the mitotic checkpoint may one day be a mainstay in our treatment of diseases such as cancer.

1.3 The aims of this thesis

This thesis is a presentation of work began in 1997 and completed in 2001. It aims to identify a component of the mitotic checkpoint in *S. cerevisiae* by identifying the locus that confers benomyl sensitivity upon two yeast strains called *mad3-1* and *mad3-2*. The thesis will go on to localise the protein translated from this locus and analyse its primary amino acid sequence. From this sequence, and the work of others, this thesis will discover Bub3p and Cdc20p interacting with Mad3p via two distinct domains within Mad3p. Further interactions between Mad3p and mitotic components and novel genes will also be identified through a two-hybrid screen. Finally the development of an assay to assay the rate of loss of chromosomes from yeast will be used to assess the possible relevance of checkpoint components to the onset of aneuploidy in yeast, and by comparison in human cancers.

Chapter 2. - Materials and Methods.

2.1. Materials.

2.1.1. General Reagents.

2.1.1.1. Chemicals.

Chemicals were purchased from the following sources, except where stated otherwise: Amersham, Fischer, Fisons, Melford Labs., National Diagnostics, Scotlab, Sigma, GibcoBRL.

2.1.1.2. Enzymes.

Restriction enzymes, DNA and other enzymes used in this work were purchased from the following sources unless otherwise stated: Boehringer Mannheim, Gibco BRL, New England Biolabs, Pharmacia, Promega, QIAGEN.

2.1.1.3. Growth Reagents.

Reagents for all growth media were purchased from: Beta Lab, Difco Laboratories, Oxoid or Sigma.

2.1.1.4. Antibiotics.

Ampicillin and Tetracycline were purchased from Sigma. G418 Sulphate was purchased from Calbiochem.

2.1.1.5 Yeast growth disrupting agents

Methyl(5-(2-Thienylcarbonyl)-1H-benimidazol-2-yl) carbamate (nocodazole in this study) and Hydroxyurea were purchased from Sigma. Benomyl was a kind gift from Dupont.

2.1.2. Growth Media.

2.1.2.1. General Information.

All growth media were autoclaved prior to use, and stored at room temperature. For solid media, 2% (w/v) agar was added prior to autoclaving.

2.1.2.2. Bacterial Media.

Table 2.1.1.

Bacterial Media

Medium	Components
Luria-Broth (LB)	1% (w/v) Bacto-tryptone* 0.5% (w/v) Yeast extract* 0.5% (w/v) NaCl pH was adjusted to 7.2 with NaOH

*purchased from Difco Laboratories

2.1.2.3. Yeast Media

Table 2.1.2.

Yeast Media.

Medium	Components
YPDA	1% (w/v) Yeast extract* 2% (w/v) Bacto-peptone* 2% (w/v) Glucose[#] 0.003% (w/v) Adenine sulphate[#]
YPD	1% (w/v) Yeast extract* 2% (w/v) Bacto-peptone* 2% (w/v) Glucose[#] 0.001% (w/v) Adenine sulphate[#]
YPGalA	1% (w/v) Yeast extract* 2% (w/v) Bacto-peptone* 2% (w/v) Galactose[#] 0.003% (w/v) Adenine sulphate[#]
YMM[a]	0.67% (w/v) Yeast Nitrogen Base w/o amino acids[#] 2% (w/v) Glucose[#]
YMGal[a]	0.67% (w/v) Yeast Nitrogen Base w/o amino acids[#] 2% (w/v) Galactose[#]

Table 2.1.2.

Yeast Media.

Sporulation	1% (w/v) potassium acetate[#] 0.1% (w/v) Bacto-yeast extract* 0.05% (w/v) Glucose (or Galactose)[#]

[a] Selective media were supplemented with nutrients from drop-out mix as required. Drop-out powder was added prior to autoclaving.

*Purchased from Difco Laboratories

[#]Purchased from Sigma Laboratories

2.1.2.4. Antibiotics.

Antibiotics were added to liquid media immediately prior to use, while for solid medium, antibiotics were added after autoclaving. All antibiotics were stored at -20°C with the exception of G418 which was stored at 4°C.

Table 2.1.3.
Antibiotics.

Antibiotic	Abbreviation	Solvent	Stock Solution (mg/ml)	Final concentration (μg/ml)
Ampicillin*	Amp	Water	100	50-100
Kanamycin*	Kan	Water	10	10
Tetracycline*	Tet	Ethanol	5	5
G418 Sulphate#	G418	Water	20	200

*Purchased from Sigma Laboratories

#Purchased from Calbiochem

2.1.2.5. Nutrients and Supplements.

Drop-out powder was prepared by mixing 2g of each of the following nutrients: Adenine; Alanine; Arginine; Asparagine; Aspartic acid; Cysteine; Glutamic acid; Glutamine; Glycine; Histidine; Isoleucine; Lysine; Methionine; Phenylalanine; Proline; Serine; Threonine; Tyrosine; Tryptophan; Uracil; Valine. If required, 4g of Leucine was included in the mix. The drop-out mix was ground with a mortar and pestle to ensure complete mixing and was used at 0.2% (w/v). The solution was adjusted to pH 7.5 by adding the appropriate amount of NaOH. All amino acids were purchased from Sigma Laboratories.

Stock solutions of individual growth supplements were sterilised by autoclaving, stored at 4°C and added to media immediately prior to use.

Table 2.1.4.

Nutrients and Supplements

Nutrient[#]	Stock solution % (w/v)	Final concentration (mg/l)
Adenine Sulphate[a]	0.2	20
Uracil[a]	0.2	20
L-Tryptophan	1.0	20
L-Histidine	1.0	20
L-Methionine	1.0	20
L-Leucine	1.0	30

[#] Purchased from Sigma Laboratories

[a] stored at room temperature.

2.1.3. Commonly Used Buffers.

Table 2.1.5.

Commonly Used Buffers.

Buffer	Components
10x TAE*	0.4M Tris-acetate, pH 7.5 20mM EDTA$^{\$}$ Unsterilised.
10x TBE*	0.45M Tris 0.45M Boric acid 10mM EDTA$^{\$}$ pH 8.0 Unsterilised.
10x PBS*	2mM KH_2PO_4 10mM Na_2HPO_4 2.5mM KCl 140mM NaCl pH 7.2 Sterilised by autoclaving.

Table 2.1.5.

Commonly Used Buffers.

Protein sample buffer	2% w/v SDS[$] 80mM Tris-Cl pH 6.8* 10% w/v Glycerol[§] 10mM EDTA[$] 0.02% v/v Bromophenol Blue* 0.1M DTT*
IP Lysis Buffer	50mM HEPES-KOH pH 7.6* 25mM KCl* 50mM NaF[$] 1mM $MgCl_2$[$] 1mM EGTA[$] 0.1% NaDeoxycholate 1mM PMSF*
10x PBS-T	2mM KH_2PO_4* 10mM Na_2HPO_4* 2.5mM KCl* 140mM NaCl* pH 7.2 10% Tween-20 Unsterilised.

Table 2.1.5.

Commonly Used Buffers.

10x TE*	0.1M Tris-HCl, pH 7.5 0.01M EDTA[$] Sterilised by autoclaving.
Yeast Lysis Buffer	2.5M LiCl* 20mM Tris-HCl pH 8.0* 4% Triton X-100[§] 62.5mM Na_2EDTA[$]
LiOAc mix*	100mM LOAc 10mM Tris-HCl pH 7.4 1mM Na_2EDTA[$]
PEG mix	40% PEG 2800 100mM LOAc* 10mM Tris-HCl pH 7.4* 1mM Na_2EDTA[$]

*Components purchased from Sigma Laboratories

[$]Purchased from Fisher Chemicals

[§] Purchased from Pharmacia Biotech

PEG 2800 purchased from Aldrich

SDS purchased from Melford Laboratories

Tween-20 purchased from USB Corporation

2.1.4. *Escherichia coli* Strains.

TG1 cells were used for cloning and the propagation of plasmid DNA. DH5α cells were used to express proteins for linkage to columns or the production of antisera.

Table 2.1.6

Bacterial strains used in this study

Strain	Genotype	Source
TG1	supE thi-1 Δ(*lac-pro*AB) Δ(*mcr*B-*hsd*SM)5 (rK- mK-) [F' *traΔ*36 *pro*AB *lac*IqZ*ΔM15*	Stratagene
DH5αF'	F', ϕ80*dlac*ZΔM15, Δ(*lac*ZYA-*arg*F) U169, *deo*R, *rec*A1, *end*A1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, λ^-, *thi*-1, *gyr*A96, *rel*A1.	Gibco BRL

2.1.5 *Saccharomyces cereivisiae* Strains

Table 2.1.7

Yeast Strains Used in this Study

Strain	Mating Type	Genotype	Reference
KH 34	**a**	*ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH 35	α	*ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH 45	**a**	*mad3-1, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH 160	**a**	*mad3-2, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH 123	**a**	*mad1Δ1::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH 125	**a**	*mad3Δ1::LEU2, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH 173	**a**	*mad3Δ2::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH 40	**a**	*cin1Δ::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
YRJ 10	**a**	*mad3Δ2::URA3, BUB3-(myc)13::G418, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	This study
YRJ 12	**a**	*BUB3-(myc)13::G418, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	This study

Table 2.1.7

Yeast Strains Used in this Study

Strain	Mating Type	Genotype	Reference
YRJ 13	**a**	*BUB3-(myc)13::G418, cdc26Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	This study
YRJ 11	**a**	*mad3-1, BUB3-(myc)13::G418, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	This study
YRJ01	**a**	*URA3 MAD3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	This study
KH232	**a**	*mad1Δ1::HIS3, BUB3-(myc)13::G418, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH234	**a**	*mad2Δ::URA3, BUB3-(myc)13::G418, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH238	**a**	*bub1Δ::HIS3, BUB3-(myc)13::G418, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH240	**a**	*bub2Δ::URA3, BUB3-(myc)13::G418, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick
KH242	**a**	*mps1-1, BUB3-(myc)13::G418, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	K. Hardwick

Table 2.1.7

Yeast Strains Used in this Study

Strain	Mating Type	Genotype	Reference
YRJ02	**a**	*mad2Δ::G418, gal4, gal80, ura3-52, his3, leu2,3-112, his3-11, trp1-901, ade2-101, URA3::GAL-lacZ, LYS2::GAL-HIS3, SPA10::GAL-URA3*	This study
YRJ03	**a**	*bub2Δ::URA3, mad3Δ 2::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100*	This study
KH231	**a**	*ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11*	K. Hardwick
YMB111	**a**	*mad1Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11*	D.M.Brady
YMB113	**a**	*mad2Δ::LEU2, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11*	D.M.Brady
YRJ111	**a**	*mad3Δ::URA3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11*	This study
YJR112	**a**	*bub1Δ::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11*	This study

Table 2.1.7

Yeast Strains Used in this Study

Strain	Mating Type	Genotype	Reference
YJR113	**a**	*bub2Δ::LEU2, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11*	This study
YJR114	**a**	*bub3Δ::TRP1, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11*	This study
CG1945	**a**	*MAT**a** ura3-52 his3-Δ200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 cyh^{r}2 LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3 URA3::GAL4$_{17\text{-}mers(x3)}$- CYC1$_{TATA}$-lacZ*	(Feilotter, Hannon et al. 1994)
Y187	α	*MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3, 112 gal4 met-gal80 URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*	(Harper, Adami et al. 1993)

2.1.6 Oligonucleotides

Table 2.1.8

Oligonucleotides used in this study

Name	Sequence	Purpose
3.3	aagttctatgtaccaaaacca	Sequencing and cloning
3.32	ttcagatctggttttccc	Sequencing and cloning
3.23	tctcagtgcttcgtccctt	Sequencing and cloning
3.34	gaaaaatttcaatatgccgtg	Sequencing and cloning
3.35	tccctttggcgtcatagg	Sequencing and cloning
3.9	gatgaggaatctaaaggt	Sequencing and cloning
3.33	ttgccctaaatttgcttc	Sequencing and cloning
3T1	aataggatccaatgtagactctaatgagtccattga	Cloning of region I mutant
3T2	atagaattcattcagaaagaaaaatttcaatatgc	Cloning of region I mutant
3.30	atgaaagcgtacgcaaag	Sequencing and cloning
3.31	tcaacgctgtggtgggta	Sequencing and cloning

2.1.7 Plasmids

Table 2.1.9

Plasmids used in this study

Plasmid Name	Genotype	Reference
pKH512	Yeast-*E. coli* shuttle vector, tpi – *MAD3*-myc, 2μm, *URA3*	pJS209 (Semenza et al, 1990)
pKH513	Yeast-*E. coli* shuttle vector, TPI – *MAD3*-myc, 2μm, *URA3*	pJS209 (Semenza et al, 1990)
pKH535	Yeast-*E. coli* shuttle vector, +500bp*MAD3*, *URA3*, CEN	This study
pCD179	Yeast-*E. coli* shuttle vector, 2μm, ARS1, MET25	Cheryl Dunbar
PCD44	Yeast-*E.coli* shuttle vector, *CEN6*, ARS1, MET25-*BUB1*	Cheryl Dunbar
PCD46	Yeast-*E.coli* shuttle vector, *CEN6*, ARS1, MET25-*MAD1*	Cheryl Dunbar
PCD47	Yeast-*E.coli* shuttle vector, *CEN6*, ARS1, MET25-*MAD2*	Cheryl Dunbar

Table 2.1.9

Plasmids used in this study

Plasmid Name	Genotype	Reference
PCD48	Yeast-*E.coli* shuttle vector, *CEN6*, ARS1, MET25-*MAD3*	Cheryl Dunbar
PCD49	Yeast-*E.coli* shuttle vector, *CEN6*, ARS1, MET25-*bub1* $_{1-210}$	Cheryl Dunbar
PCD50	Yeast-*E.coli* shuttle vector, *CEN6*, ARS1, MET25-*bub1* $_{1-367}$	Cheryl Dunbar
PCD51	Yeast-*E.coli* shuttle vector, *CEN6*, ARS1, MET25-*bub1* $_{1-608}$	Cheryl Dunbar
pCD45	Yeast-*E.coli* shuttle vector, *CEN6*, ARS1, MET25-*BUB3*	Cheryl Dunbar
pBUB3-GST	*E. coli* expression vector, GAL4-*BUB3*-GST, *ori*	K. Hardwick
pMAD3-GST	*E. coli* expression vector, GAL4-*MAD3*-GST, *ori*	K. Hardwick
pRS312	Yeast-*E. coli* shuttle vector: Multiple cloning site; *lacZ*α; Amp^R; *CEN6, ARSH4*; *URA3*	Sikorski and Hieter, 1989.

Table 2.1.9

Plasmids used in this study

Plasmid Name	Genotype	Reference
pLH68	Yeast-*E. coli* suttle vector: *CDC20*-$(ha)_3$; *URA3*; *CEN6*	This study
pRJ68	Yeast-*E. coli* suttle vector: *CDC20*-$(ha)_3$; *LEU2*; *CEN6*	This study
pRJ21	Yeast-E. coli shuttle vector: $MAD3_{(123AAA127)}$	This study
pRJ22	Yeast-E. coli shuttle vector: $MAD3_{(E322K)}$	This study
pACTII	Yeast-*E. coli* shuttle vector: *ADH1* promoter sequence, Gal4AD sequence (aa 768-881), HA-epitope sequence; Amp^R; 2μ; *LEU2*.	Clontech.
pAS2	Yeast Two-hybrid vector containing the ADH1 promoter, *TRP1* gene, *bla* AMPr gene and the GAL4 DNA binding domain.	(Harper, Adami et al. 1993)
pACTIIst	Modified pACTII. Two-hybrid prey plasmid containing linker encoding multiple STOP codons.	(Fromont-Racine, Rain et al. 1997)

2.1.8 Antisera

Table 2.1.10.
Antisera.

Antibody	Description	Origin
Anti-Mad3p	Rabbit polyclonal antibodies raised against full length Mad3p fused to Glutathione S-Transferase 1:1,000 dilution for Western Blots. 5µl per immunoprecipitation.	Hardwick *et al* 2000
Anti-Bub1p	Rabbit polyclonal antibody raised against full length Bub1p fused to Glutathione S-Transferase 1:1,000 dilution for Western Blots 5µl per immunoprecipitation	Hardwick *et al* 2000
Anti-Mad2p	Sheep polyclonal antibody raised against full length Mad2p fused to Glutathione S-Transferase 1:1,000 dilution for Western blots 5µl per immunoprecipition	Brady and Hardwick 2000

Table 2.1.10.

Antisera

Antibody	Description	Origin
Anti-Mad1p	Rabbit polyclonal antibody raised against full length Mad1p fused to Glutathione S-Transferase 1:1,000 dilution for Western blots 5µl per immunoprecipition	Hardwick *et al* 1997
Anti-HA	The Mouse monoclonal antibody, 12CA5, was raised against the haemagglutinin HA-1 protein epitope of the influenza virus. 1:1,000 dilution for Western blots. 5µl per immunoprecipitation.	Santa Cruz Laboratories
Anti-MYC	The 9E10 Rabbit polyclonal antibody raised against the c-myc protein 1:1,000 dilution for Western blots 5µl per immunoprecipitation.	Santa Cruz Laboratories
Anti-Rabbit IgG-HRP	Anti-rabbit IgG Horse Radish Peroxidase linked whole antibody (from donkey). 1:5,000 dilution for Western Blots.	Amersham.

Table 2.1.10.

Antisera

Antibody	Description	Origin
Anti-Sheep IgG-HRP	Anti-sheep IgG Horse Radish Peroxidase linked whole antibody (from ? 1:5,000 dilution for Western Blots	SAPU
Anti-Mouse IgG-HRP	Anti-mouse IgG Horse Radish Peroxidase linked whole antibody (from sheep). 1:5,000 dilution for Western Blots.	Amersham.

2.2 Methods

2.2.1 Analysis of Strains

2.2.1.1 Growth of bacterial strains

E. coli strains were routinely grown at 37°C in rich LB (Table 2.1.1.). To maintain selection for plasmid DNA the transformed bacteria were grown in medium containing the appropriate antibiotic or frozen at –80°C (Table 2.1.3).

2.2.1.2. Growth of yeast.

Yeast strains were routinely grown at 30°C on YPDA or YPD (Table 2.1.2). To maintain selection for plasmid DNA, and/or for a metabolic reporter gene inserted on the genome, cells were grown at 30°C on YMM or YMGal (Table 2.1.2) supplemented with the appropriate drop-out powder or nutrients (Section 2.1.2.5.). Temperature-sensitive yeast strains were routinely grown at 23°C, in the appropriate medium for that strain.

2.2.1.3 Preservation of bacteria.

E. coli strains were stored for up to two weeks on solid medium at 4°C. Strains were stored indefinitely at -80°C in 15% (v/v) glycerol. A stationary culture of the strain to be frozen was grown in appropriate medium. Eight hundred microlitres of culture was mixed with an equal volume of sterile 30% (v/v) glycerol and snap-frozen on dry ice.

2.2.1.4 Preservation of yeast

Saccharomyces cerevisiae strains were stored for up to two weeks on solid medium at 4°C. Strains were stored indefinitely at -80°C in 30% (v/v) glycerol. A colony was picked from an overnight plate of appropriate medium and mixed in 1ml 30% (v/v) glycerol. After a 30 minute incubation at room temperature the mixture was placed in a -80°C freezer where it was stored indefinitely.

2.2.1.5 Growth of diploid yeast strains.

Diploid yeast cells of the strain to be sporulated were grown on solid medium. Cells were picked from the solid medium and grown in liquid sporulation medium (Table 2.1.2.). Cells were examined microscopically to determine if sporulation and tetrad formation had occurred; usually 3-4 days.

2.2.1.6 Tetrad dissection.

Upon successful sporulation and tetrad formation, 1ml of the sporulation mixture was spun down in a microfuge at 10,000g for 30 seconds and resuspended in 200ml of sterile distilled water. To this suspension, 3-5µl of β-glucuronidase (10 units/µl stock solution) was added, mixed gently and incubated at room temperature for 30-60 minutes. Cell wall digestion was assayed microscopically during the incubation period. Tetrads were dissected using a Singer MSM system micro-manipulator, on YPDA (Table 2.1.2.) and allowed to grow to visible colonies. The tetrads were then replica plated onto YMM plates (Table 2.1.2.) containing the relevant amino acids for selection of markers in the genome.

2.2.1.7 Transformation of bacterial strains

E coli cells were made competent using the Chung method (Chung, Niemela et al. 1989). DH5α or TG1 competent cells were thawed on ice for 10 minutes. 1µl of DNA (10µl of ligation) was added to a chilled 1.5ml Eppendorf tube and to this 50µl of the thawed competent cells was added. The mixture was incubated on ice for a further 10 minutes then transferred directly to a 37°C water bath and incubated for 5 minutes. 150µl of LB media was added to the mixture and incubated at 37°C for 30 minutes. After incubation the mixture was plated onto selective media and grown overnight at 37°C.

Colonies were then picked and the plasmid recovered by Mini or Maxi prep.

2.2.1.8 Transformation of yeast strains

The strain to be transformed was grown overnight in 50ml of the appropriate YPDA or selective media to attain an OD_{600} or between 0.3-0.5 the following morning. The log phase culture was centrifuged at 3500rpm for 1 minute and washed and transferred to a 1.5ml Eppendorf tube in 1ml LiOAc mix (Table 2.1.5). This mixture was then centrifuged again at 3500rpm for 1 minute and resuspended in 500µl of LiOAc mix. This last centrifugation step was repeated and the pellet was again resuspended in 500µl of LiOAc mix.

1-2µl of the DNA to be transformed was added to 1.5ml Eppendorf tubes to which was also added 15µl of salmon sperm DNA. To this mixture was added 100µl of the yeast mixture. 700µl of PEG mix (Table 2.1.5) was added to the cell/DNA mixture and mixed using a 1ml blue Eppendorf pipette tip. This final mixture containing the DNA, cells and PEG was incubated at room temperature for 30 minutes.

The resulting mixture was heat shocked for 15 minutes in a 42°C water bath. After heat shock the mixture was centrifuged at 1000rpm for 30 seconds and the supernatant was removed. The pellet was resuspended in 200µl of sterile water and plated onto selective media and grown at the appropriate temperature for 3 days.

Colonies were picked and streaked onto selective media and tested for transformation by PCR or by recovery of the plasmid (2.2.1.9) and restriction endonuclease digestion.

2.2.1.9 Isolation of DNA from yeast

DNA was isolated from yeast using the Ward method as follows (Ward 1990). The yeast strain was grown overnight to attain an OD_{600} of between 0.3-0.5 the following morning. 1.5ml of the culture was centrifuged at 13000rpm for 1 minute and the supernatant was removed. 200µl of yeast Lysis Buffer (Table 2.1.5) was added to the top of the pellet. To this an equal volume of phenol/chloroform (1:1) and 0.2g of glass beads were added (0.45 - 0.50mm Braun glass beads).

The mixture was vortexed for 2 minutes and centrifuged for a further 1 minute. The top phase was collected and ethanol precipitated by the addition of sodium acetate to 0.2M followed by 2 volumes of ethanol, chilling the mixture at -20°C for 10 minutes and centrifuging at 13000rpm for 10 minutes. The resulting precipitate was resuspended in 20µl TE.

2.2.1.10 Microcolony assay of yeast strains

Thin streaks of yeast were plated onto YPD media and grown overnight. The following day, single cells of a uniform size were manipulated by a needle into lines on each plate.

The colony sizes were noted at regular time intervals for up to 12 hours resulting in a measurement of the rate of division of each individual cell. This was performed for over 100 cells for each strain analysed and repeated at least three times.

2.2.1.11 Yeast Two-Hybrid Screen.

Yeast two-hybrid screens were performed using the method of Fromont-Racine *et al.* (Fromont-Racine, Rain et al. 1997).

2.2.1.11.1 Bait Construction.

The two-hybrid bait fusion was constructed in a plasmid derived from pAS2 (Table 2.1.9.) using standard recombinant DNA procedures (Section 2.2.3). All bait constructs were transformed into a *mad3Δ* yeast strain (KH173) to confirm their

functionality by complementation. Each was then tested for the expression of the fusion protein

The auto-activation properties of each bait construct were tested either by "mini-screen", performed essentially as for a full screen (Section 2.2.1.11.2), but with only approximately one tenth of the number of FRYL library-containing cells used in a full screen. For either case, diploid cells were plated onto YMM -LW and YMM -LWH medium (Table 2.1.2) and the plates incubated at 30°C for 4 days. A comparison of the number of clones able to grow on YMM -LWH (*i.e.* diploid cells containing both bait and prey plasmids and supporting a two-hybrid interaction) to the number of clones able to grow on YMM -LW (*i.e.* diploid cells containing both bait and prey plasmids) gives an indication of the auto-activating potential of the bait construct and of the number of positive clones which could be expected from a full coverage of the FRYL library.

2.2.1.11.2 Construction of the FRYL Library.

The FRYL library used in the two-hybrid screens described in this work was constructed by Micheline Fromont-Racine in the laboratory of Pierre Legrain (Fromont-Racine *et al.*, 1997) as follows.

Genomic DNA was sonicated and treated with three modification enzymes (Mung bean nuclease, T4 DNA polymerase and Klenow) to produce blunt ended fragments. Adaptors were ligated to these fragments, producing a 3' overhang which and were then ligated into the pACTIIst plasmid (Table 2.1.9) which had previously been digested with *Bam*HI and "filled-in" with dGTP by the Vent (Exo^-) polymerase. Library was transformed into *E. coli* cells, transformant colonies were scraped from the plates, pooled and frozen. Cells were stored at -70°C. Library DNA was extracted from these bacterial cells and transformed into Y187 cells (Section 2.2.1.8)
. Cells were scraped from the transformation plates, pooled and aliquoted. Aliquots (1ml) were stored in 15% (v/v) glycerol at -70°C.

2.2.1.11.3 Mating and Collection of Diploids.

The bait plasmid was transformed (Section 2.2.1.8) into the carrier yeast strains, CG1945 (Table 2.1.7) and propagated on YMM -W medium (Table 2.1.2.3). Bait cells were grown to an OD_{600} of 0.8-1.0 units. An aliquot of Y187 cells containing the FRYL library was thawed on ice, inoculated into 20ml of YPDA + Tet (Tables 2.1.2.3 and 2.1.3) and incubated at 30°C for 15 minutes, with gentle shaking (approximately 120 rpm). Bait cells equivalent to 80 OD_{600} units (approximately 8 x 10^8 cells) were mixed with the library-containing cells, and the cells concentrated onto twelve Millipore filters (ϕ 45mm filters, 0.22μm from 3MM) Each filter was washed with 5 ml of fresh YPDA + Tet medium and incubated for 5 hours on solid YPDA + Tet medium at 30°C. Cells were collected by washing from the filters with YMM -LWH (Table 2.1.2.3) into a total volume of approximately 25ml. Collected cells were mixed thoroughly and 50μl removed for control plates. (These cells were diluted to 1:1000 by serial dilution in YMM, and 50μl plated onto each of YMM -L, YMM -W and YMM -LW medium (Table 2.1.2.3). Plates were incubated at 30°C for 2 days (see 2.2.1.11.4 for calculation of mating efficiency and coverage of the library)).

The mated cells were spread onto YMM -LWH + Tet medium (Tables 2.1.2.3 and 2.1.3) at about 250μl per plate, and incubated at 30°C for 4 days.

2.2.1.11.4 Calculation of Mating Efficiency and Library Coverage.

The number of colonies growing after two days on each of the control plates was counted. Those colonies growing on YMM -L represent cells (haploid and diploid) which contain library plasmid DNA, while those growing on YMM -LW represent diploid cells containing both bait and prey (library) plasmid DNA.

Formula to calculate the mating efficiency:

$$\text{Mating efficiency (\%)} = \frac{\text{Number of colonies on YMM -LW}}{\text{Number of colonies on YMM -L}} \times 100$$

Formula to calculate the library coverage:

Diploids screened = Colonies on YMM -LW x Dilution factor x Volume of culture.

In order to cover the full library, 1.5×10^7 diploids must be screened.

2.2.1.11.5 Filter-lift assay.

Filter-lift assays were performed as described in Transy and Legrain (Transy and Legrain 1995).

Filter-lift assay solution (below) for the detection of β-galactosidase activity was prepared fresh from stock solutions immediately prior to use. Cells were transferred to Hybond-C Extra filters (Amersham), and the filters immersed in liquid nitrogen for 10 seconds. Filters were placed (cell-side up) onto Whatman 3MM paper soaked in assay solution, incubated at 30°C and examined at regular intervals (usually every two hours). The reaction was stopped by transferring the filter to Whatman 3MM paper soaked in STOP solution (see below) for 60 seconds, and then to Whatman 3MM paper soaked in sterile water for 60 seconds.

- Z-buffer: 100mM $NaPO_4$, pH 7.5
  10mM KCl
  1mM $MgSO_4.7H_20$
  Sterilised by autoclaving.
- Assay solution: 0.27% (v/v) β-mercaptoethanol
  0.04% (w/v) X-Gal (in dimethyl formamide)
  Prepared in Z-buffer.
- STOP solution: 1M Na_2CO_3
  Sterilised by autoclaving.

2.2.8.7. Analysis of Positive Colonies from Two-Hybrid Screens.

Colonies which passed all test criteria *i.e.* His+ and LacZ+ (in filter-lift assay) were analysed to identify the library plasmid responsible for the two-hybrid interaction. Plasmid DNA was rescued from the yeast cells by the method described in Section 2.2.1.9, and transformed (Section 2.2.1.8) into TG1 cells (Table 2.1.6). These cells were plated onto LB plates containing ampicillin (Tables 2.11 and 2.1.3) which allowed growth of only those cells carrying the library plasmid. The plasmid DNA was propagated in these cells and prepared by the method described. Insert size was determined following restriction digestion with *Bam*HI (which cuts in the library adaptor sequence at both ends of the insert) and agarose gel electrophoresis (Section 2.2.3.3). The fusion between the vector sequence and the genomic DNA insert was determined by sequencing as described in Section 2.2.3.7. The identity of the genomic insert was determined by searches of the *Saccharomyces* Genome Database (SGD) Section 2.2.4.

2.2.2 Analysis of proteins

2.2.2.1 Western analysis of proteins

Samples of yeast for Western analysis were pelleted at 5000rpm for 1 minute and the mass was equalised on scales in 1.5ml Eppendorf tubes. The cells were washed once in H_2O and pelleted once again. To the washed pellet was added 100µl Protein Sample Buffer containing 100mM DTT and 1mM PMSF. To this was added 50µl of Zirconium beads and the mixture was bead-beaten at 4°C for 1 minute at 5000rpm using a Mini-Beadbeater™ from Biospec. The lysed cells were then centrifuged at 13000rpm for 1 minute at 4°C.

The samples were boiled and loaded onto a Polyacrylamide gel to which a current of 180V was applied for 45 minutes. The finished gel was placed between 3MM paper and a Nitrocellulose membrane and a current of 20V was passed through this sandwich for 2 hours using the Hoefer EPS 2A200 system.

Upon successful transfer of the protein from the gel to the nitrocellulose membrane the membrane was temporarily stained for protein with Ponceau Red. This allowed the identification of the molecular weight markers which were then permanently marked with an ink pen. The Ponceau Red was washed off with water and the membrane was incubated in Blocking solution (PBS-T containing 5% milk powder (from SMA)) for 10 minutes.

The membrane was then incubated in primary antibody diluted in blocking solution to the appropriate dilution and incubated at room temperature for 1 hour. After incubation with primary antibody the membrane was washed in PBS-T for 5 minutes. This washing was repeated twice, after which the membrane was suspended in Horse Radish Peroxidase linked secondary antibody, also in blocking solution. This incubation lasted for 1 hour after which the membrane was washed in PBS-T and the activity of the horse radish peroxidase enzyme was detected using the ECL method.

2.2.2.2 Immunofluorescence

The strain to be analysed by immunofluorescent microscopy incubated in media containing 3.7% formaldehyde for 30 minutes. After 30 minutes the cell samples were centrifuged at 5000rpm for 1 minute and resuspended in 1ml 0.1M potassium phosphate pH 7.4 containing 1.2M sorbitol (KPi) to maintain the osmotic balance of the cells. This cell solution was centrifuged again and resuspended in 1ml KPi. The cells were then spheroplasted to aid access to the cell by antibodies using zymolyase, an enzyme extracted from *Arthrobacter luteus*. To spheroplast, the cells were suspended in KPi containing 250mg/ml 20T zymolyase for 10 minutes at 30°C then centrifuged at 5000rpm for 1 minute. The pellet of cells was washed twice in Kpi followed by one wash in PBS containing 1.2M sorbitol ((PBSS)(purchased from Fisher Laboratories)) and resuspended in 1ml of this solution.

Slides were prepared by adding 0.1mg/ml polylysine (purchased from Aldrich) to the slide using a 200µl Eppendorf pipette and incubating for 2 minutes. The slide was then washed with PBSS. The prepared slide was then stored in a humid chamber.

The cell suspension was spotted onto the prepared slides and incubated in the humid chamber for 10 minutes. The remaining liquid on the slide was removed using a pipette and the slides were washed with PBSS. An additional fixation step was used to aid visualisation of the cells. The slides, now with attached cells, were plunged into methanol at –20°C for 1 minute, then transferred immediately to acetone again at –20°C for a further 30 seconds.

The slides were then washed twice using PBS and incubated in Blocking solution (PBS-T containing 5% milk powder) for 30 minutes in the humid chamber. Primary antibody was added to the desired dilution in Blocking solution and incubated with the cells for 2 hours in the humid chamber. The slides were washed twice in blocking solution and incubated in the suitable secondary antibody at the desired dilution in blocking solution for 1 hour. The secondary antibody was incubation was performed in the dark to avoid bleaching of the flourescence. Upon incubation in secondary antibody the slides were then washed in blocking solution. Mounting solution containing DAPI stain was spotted onto the slides to allow visualisation of the DNA. The cells were viewed using a Ziess Axioscop fluorescence microscope and the images were analysed using IPLabs software.

2.2.2.3 Expression of GST fusion proteins

Bacteria were grown overnight containing 75µg/ml ampicillin. 4 litres of LB media containing 75µg/ml was inoculated with 100-200mls of this overnight to attain an OD_{600} of 0.1-0.2. This culture was grown until an OD_{600} of 0.8-1.0 was attained and to this was added IPTG to 0.1mM. This culture was then grown for a further 3 hours at room temperature. The cells were collected by centrifuging at 3000rpm for 5 minutes and the pellet was frozen in liquid nitrogen.

The frozen pellet was then ground in a pestle and mortar in liquid nitrogen and allowed to warm to room temperature to prevent ice crystal formation when the buffer was added. 5 volumes of PBS containing 0.5% Tween-20, 1mM PMSF and 1M NaCl was added to the ground pellet and the solution was sonicated for 1 minute at room temperature. All subsequent procedures were performed at 4°C. The sonicated solution was placed at 4°C and to this was added 10mM DTT. This solution was then centrifuged at 35,000rpm in a Beckman 50.2 Ti rotor for 1 hour.

The supernatant was collected and added to a 10ml glutahtione agarose column (Sigma G-4510) over a period of 3-4 hours. Once the supernatant was passed over the column the column was washed with 100mls PBS containing 0.05% Tween-20, 0.5mM DTT and 0.25M KCl. The effluent was monitored for protein using the Bradford assay. Once no protein was found in the effluent, the column was washed with 2 volumes of PBS containing 5mM DTT and 0.25M KCl.

The column was eluted in 50mM Tris pH 8.1 containing 0.25M KCl and 5mM reduced glutathione (Sigma). The elution was performed in 1ml fractions and these fractions were assayed for protein using the Bradford assay. The peek fractions were pooled together and dialysed into 50mM Hepes pH 7.6 containing 100mM KCl and 30% glycerol. This method yielded a protein concentration of 20-50mg/ml.

2.2.2.4 Coupling of proteins to Affigel 10

The Affigel 10 (purchased from Biorad) slush was stored at -20°C in isopropanol. The desired amount of gel was collected in a centrifuge tube and pelleted at 3,500rpm. The pellet was then resuspended in ice cold coupling buffer; 50mM Hepes pH 7.6 and 100mM KCl. This step was repeated three times. After the

supernatant was removed for the final time, a solution of the coupling buffer containing 5mg/ml of the protein to be coupled was mixed with the beads in a ratio of 1:1. The mixture was then pelleted again at 3,500rpm and a 5µl sample of the supernatant was taken, corresponding to the zero time point. The slurry was allowed to mix at 4°C, and 25µl samples were taken every 5 minutes. The samples were assayed for protein using the Bradford assay and once 80% of protein from the initial 5µl zero time point was detected the coupling reaction was stopped by adding 1M Tris pH 7.5 to a final concentration of 50mM. The column was then either used immediately or stored at -20°C in 50% glycerol.

2.2.2.5 Immunoprecipitation

Samples to be anaylsed by immunoprecipitation(IP) were centrifuged at 5000rpm for 1 minute and the supernatant removed.. The pellet was washed in water and 500µl of IP lysis buffer (Table 2.1.5) was added to the pellet. To this was added 50µl of glass beads (0.45 - 0.50mm) and the mixture was bead-beaten for 2 minutes. DTT was added to the lysate to a final concentration of 500mM, the lysate was centrifuged for at 13000rpm for 10 minutes at 4°C. The supernatant was removed and centrifuged again at 13000rpm for 3 minutes. This centrifugation step was repeated until no pellet was present, usually 2 repeats were required. A 20µl sample of the lysate was taken and stored at –80°C. This was used as the total imput of protein in the immunoprecipitation.

The primary antibody bound to Protein A beads was added to the lysate and incubated at 4°C for 3 hours. The mixture was rotated to ensure even mixing of the Protein A beads/primary antibody slurry. After 3 hours the mixture was centrifuged at 1000rpm for 1 minute and the supernatant collected and stored at –80°C. This was used as the supernatant sample when the immunoprecipitation was analysed using Western blotting. The remaining bead/antibody slurry was washed in IP buffer three times.

The three stages of the immunoprecipitation – total fraction, supernatant and beads – were subjected to the Western blotting protocol.

2.2.2.6 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE).

All SDS-polyacrylamide gels were run using two 'sealed' 16 x 16 cm glass plates separated by 1.5mm spacers and using 14 well combs. The resolving gel solution was prepared, poured between the plates and overlaid with water-saturated butanol. The gel was allowed to set at room temperature, the butanol was washed off with sterile, distilled water and the stacking gel was poured. After polymerisation was complete, the seal was removed from the plates, the comb was gently taken out and the wells were washed with distilled water. The plates were firmly fixed in the electrophoresis apparatus and the chambers filled with 1x protein gel running buffer. Protein extracts were mixed with an equal volume of 2x SDS loading buffer, heated to 96°C for 5 minutes and centrifuged at 14,000 rpm for 60 seconds. Samples were loaded onto the gel, and run until the bromophenol blue dye-front had reached the bottom of the resolving gel.

2.2.3 Anaylysis of DNA molecules

2.2.3.1 Amplification of DNA using the Polymerase Chain Reaction (PCR).

Specific regions of DNA were amplified using the polymerase chain reaction (PCR). Template DNA was either a small quantity of plasmid DNA or yeast genomic DNA using commercially produced primers. The Pfu polymerase from Stratagene was used (Catalog number 600153) A typical 50µl reaction using plasmid DNA as template was as follows:

10x Polymerase Buffer	5µl
100mM $MgCl_2$*	1µl
2.5mM dNTPs (dATP, dCTP, dGTP, dTTP)	5µl
Oligonucleotides primer 1 (50pmol/µl)	1µl
Oligonucleotides primer 2 (50pmol/µl)	1µl
Template DNA (10ng/µl)	1µl
DNA polymerase (2U/µl)	0.5µl
Sterile distilled water	35µl

* $MgCl_2$ concentration was titrated to 2, 4 or 6 mM as required.

All PCRs were carried out in a Hybaid Thermal Reactor or in a PTC-100 Hot Lid reactor (Genetic Research Instrumentation Ltd), programmed according to the length of the desired product, and the annealing temperature of the oligonucleotide primers being used. A programme is given below, with typically 30 cycles used.

Step 1. Denaturation: 95°C for 30 seconds

Step 2. Primer annealing: 40-60°C* for 30 seconds

Step 3. Extension: 72°C for 60-180 seconds.

* estimated for oligonucleotides up to 20 bases in length using the formula:

Annealing Temperature = {4 (G + C) + 2 (A + T)} - 5°C. A gradient PCR machine was used to refine the annealing temperature by varying the annealing temperature over a range of 10°C from the estimated temperature.

2.2.3.2 Purification of PCR products

DNA fragments generated by PCR were purified from oligonucleotide primers, nucleotides, polymerases and salts using QIAquick PCR purification columns (QIAGEN) as per the manufacturers' protocols. From a 50µl reaction, purified DNA was typically eluted in 30µl of sterile, distilled water and stored at -20°C.

2.2.3.3 Agarose Gel Electrophoresis.

DNA fragments produced by restriction endonuclease digest or generated by amplification in PCR were analysed in 1.0-1.5% (w/v) agarose gels (agarose purchased from Roche). Gels were prepared by melting agarose in 1x TAE buffer (Table 2.1.5) and adding ethidium bromide to a final concentration of 0.5µg/ml. Samples to be analysed were loaded directly using Ficoll loading buffer.

10x Ficoll loading buffer:	20% (w/v) Ficoll
	1% (w/v/) SDS
	0.25% (w/v) Bromophenol blue
	0.25% (w/v) Xylene cyanol
	100mM EDTA

2.2.3.4 Isolation of DNA from Agarose Gel Slices.

To isolate and purify specific DNA bands from agarose gels (agarose purchased from Roche), the QIAquick Gel Extraction kit (QIAGEN) was used as per the manufacturers' protocols. DNA fragments were separated by agarose gel electrophoresis (Section 2.2.3.3) and the bands visualised on a UV transilluminator. The band to be purified was excised with a clean razor blade, and purified. DNA was typically eluted in 20µl of sterile, distilled water and stored at -20°C.

2.2.3.5 Ligation of DNA Molecules.

Ligations were typically performed in a final volume of 10µl, containing 0.5-1.0 µg total DNA, 1x ligation buffer, 1mM ATP and 0.5 units DNA ligase (NEB Laboratories). Vector and insert DNA were present in approximately a 1:1 or 1:2 ratio. Reactions were allowed to proceed at room temperature for up to 2 hours before being stopped by 15 minutes incubation at 70°C. Between 0.5-2 µl of the reaction mix was used to transform *E. coli* TG1 cells (Section 2.1.4).

2.2.3.6 Restriction Digests of DNA.

Restriction endonuclease digestion of DNA was typically performed in volumes of 20-50µl. These contained the requisite quantity of DNA and the appropriate buffer (as supplied by the manufacturer) at 1x concentration. Between two and five units of restriction enzyme were added, with the restriction enzyme volume kept below 10% of the total reaction volume. The digest was incubated at the temperature recommended by the supplier, typically for a period of 2 hours. The products of the digestion were either analysed directly by agarose gel electrophoresis (Section 2.2.3.3) and purified (Section 2.2.3.4) prior to further manipulations. All restriction endonuclease enzymes were purchased from New England Biolabs.

2.2.3.7 Automated Sequencing of DNA molecules using PCR

DNA molecules were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase. 2μl of a Plasmid Preparation was typically used in each reaction using the following programme:

90°C for 30 seconds

50°C for 15 seconds

60°C for 240 seconds

Repeat for 25 cycles

The reactions were precipitated using ethanol and sequenced using an ABI PRISM 300 machine which detects the different fluorescent signals from the different dye linked terminating bases.

2.2.4 Computer Analyses.

Yeast database searches were performed on the *Saccharomyces* Genome Database (SGD) network (http://genome-www.stanford.edu/Saccharomyces/).

Protein database searches were performed on the NCBI network server using the BLAST algorithm (Altschul *et al.*, 1990); (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast?Jform=0).

Multiple protein sequence alignments were performed using the ClustalW server (http://www.clustalw.genome.ad.jp/)

Sequence identities and similarities were identified using the BOXSHADE 3.21 server (http://ulrec3.unil.ch/software/BOX_form.html).

Chapter 3 - *MAD3* encodes a component of the mitotic checkpoint which is ubiquitously expressed in the nucleus of *Saccharomyces cerevisiae*

3.1 Introduction

Checkpoints ensure the fidelity of the cell cycle of an organism. A checkpoint component does not have an essential role in the cell cycle but instead is only required when a problem arises as the cell proceeds through a stage of the cell cycle (Weinert and Hartwell 1988). The spindle checkpoint responds to errors in the formation of a bipolar spindle and the bipolar attachment of sister chromatids to that spindle. As the mitotic spindle is composed of interdigitating arrays of microtubules, any drug that affects microtubules will also affect the stability of the bipolar spindle and activate the checkpoint. One such drug is benomyl.

High concentrations of benomyl are toxic to yeast cells, indeed the drug is used by the agricultural industry as an anti-fungal agent (Sims, Mee et al. 1969). However, lower concentrations of benomyl are not permanently detrimental to a population of yeast but slow its growth (Li and Murray 1991). The growth rate is slowed due to an arrest in the progress of the cell cycle caused by activation of the spindle checkpoint (Li and Murray 1991). Although this study uses benomyl; or it's close relative, nocodazole; the spindle checkpoint responds to many errors in the correct attachment of sister chromatids to the bipolar spindle (Hardwick, Li et al. 1999).

To ascribe a spindle checkpoint function to a locus, a mutant in that locus must fulfil two criteria. Firstly, it must be sensitive to perturbations in the mitotic spindle, such as those caused by benomyl, and secondly, it must divide faster on benomyl than a wild type strain. The aim of this chapter is to prove that *MAD3* encodes a gene whose product is involved in the spindle checkpoint of the yeast *Saccharomyces cerevisiae*.

The components of the spindle checkpoint would be active during mitosis when the checkpoint is required. This suggests that their expression pattern may be cell cycle regulated with a peak of expression during metaphase and an increase in stability in the presence of nocodazole. Indeed, such an expression pattern is seen in higher eukaryotes for hBub1 and hBubR1 (Jablonski, Chan et al. 1998). Work in yeast suggests the checkpoint proteins are expressed at a constant level throughout the cell

cycle (Hwang, Lau et al. 1998; Brady and Hardwick 2000). In this chapter an antibody specific to *Saccharomyces cerevisiae* Mad3p is raised and used to localise Mad3p in yeast and to trace the Mad3p levels in the cell cycle. A Bub3p specific antibody is also produced although it was not used in this study.

3.2 YJR013c encodes the *MAD3* gene in *S. cerevisiae*

3.2.1 YJR013c is tightly linked to the *mad3-1* and *mad3-2* mutations

Two mutants, *mad3-1* and *mad3-2*, were originally identified in 1991 and 1995 respectively, in screens for strains sensitive to benomyl (Li and Murray 1991) (Hoyt, M.A per comm). A plasmid containing the YJR013c ORF was shown to rescue the benomyl sensitivity phenotype by the Murray laboratory. To investigate whether this ORF encoded the *MAD3* gene, or was a suppresser of the phenotype, the *URA3* gene was integrated 500bp upstream of YJR013c creating strain YRJ01 in this work. This strain was then mated with *mad3-1* and *mad3-2*. The resultant diploids were sporulated and the benomyl sensitivity and uracil prototrophy of 16 tetrads for each diploid were assayed by replica plating the spores onto plates containing 10μg/ml or 12.5μg/ml of benomyl and plates lacking the amino acid uracil respectively. In the 32 tetrads analysed benomyl sensitivity did not co-segregate with growth on -uracil plates for either the *mad3-1* locus or the *mad3-2* locus. A random pattern of segregation would suggest the YJR013c and *mad3* mutant loci were unlinked. The co-segregation of benomyl resistance with uracil prototrophy shows non-random segregation suggesting a linkage between the loci.

3.2.2 Sequencing of the YJR013c ORF in *mad3-1* and *mad3-2* mutations confirms mutations in both strains.

To confirm the benomyl sensitive phenotype seen in the *mad3-1* and *mad3-2* strains was due to mutations in YJR013c, the open reading frame in each strain was sequenced (Section 2.2.3.7). *mad3-1* was found to harbour a point mutation at nucleotide position 1144, changing glutamate 382 to lysine (Fig.3.1). The *mad3-2* mutant had a stop codon at nucleotide position 261, terminating the translation of the protein at amino acid 87 (Fig.3.1). The mutants were sequenced by making a genomic preparation from each strain (Section 2.2.1.9) and using this as a template for PCR amplification of the reading frame including 500bp upstream and downstream from the reading frame. Primers within the YJR013c ORF were used to sequence the mutants on both strands (Table 2.1.8). Sequencing was performed as described in Section 2.2.3.7. The caveats linked to sequencing of a PCR product have to be recognised; the introduction of a mutation during the initial amplification of the genomic DNA or the presence of a deletion of the genomic DNA, preventing primer annealing. No deletion of the reading frame was detected and a wild type yeast strain was used as a control for the reaction. At least ten colonies were purified using the method described in section 2.2.1.9 for *mad3-1* and *mad3-2* and all had the mutations at the nucleotide positions stated. It can be concluded from this that the PCR amplification step did not introduce new mutations into the sequence.

3.2.3 YJR013c mutants are sensitive to the microtubule depolymerising drug benomyl.

To confirm the benomyl sensitivity conferred by YJR013c loss of function, two disruptions of the *MAD3* locus were made. *mad3Δ1* was made by inserting the *LEU2* gene into the *MAD3* open reading frame, between amino acids 236-388. The second disruption, *mad3Δ2*, was constructed by replacing amino acids 60-480 with the *URA3* gene. *mad3Δ1*, *mad3Δ2*, the previously characterised *mad1Δ* (Hardwick and Murray 1995) mutant and a *cin1Δ* were stamped onto YPDA, YPDA + 10μg/ml benomyl and YPDA + 12.5μg/ml benomyl plates. The *cin1Δ* is included as a control to show the

Figure 3.1

```
1   MKAYAKKRI SYMPSSPSQNVI NFEEI ETQKENI LPLKEGRSAAALSKAI HQPLVEI NQVK  60

    SSFEQRLI DELPALSDPI TLYLEYI KWLNNAYPQGGNSKQSGMLTLLERCLSHLKDLERY 120
                          * mad3-2

    RNDVRFLKI WFWYI ELFTRNSFMESRDI FMYMLRNGI GSELASFYEEFTNLLI QKEKFQY 180

    AVKI LQLGI KNKARPNKVLEDRLNHLLRELGENNI QLGNEI SMDSLESTVLGKTRSEFVN 240

    RLELANQNGTSSDVNLTKNNVFVDGEESDVELFETPNRGVYRDGWENFDLKAERNKENNL 300

    RI SLLEANTNLGELKQHEMLSQKKRPYDEKLPI FRDSI GRSDPVYQMI NTKDQKPEKI DC 360

    NFKLI YCEDEESKGGRLEFSLEEVLAI SRNVYKRVRTNRKHPREANLGQEESANQKEAEA 420
                           K mad3-1

    QSKRPKI SRKALVSKSLTPSNQGRMFSGEEYI NCPMTPKGRSTETSDI I SAVKPRQLTPI 480

    LEMRESNSFSQSKNSEI I SDDDKSSSSFI SYPPQR 515
```

Figure 3.1 - Mutations within the YJR013c open reading frame in *mad3-1* and *mad3-2*. The terminal amino acid found in the *mad3-2* strain is marked at position 87. The *mad3-1* point mutation is marked at position 382.

phenotype of a structural component of the mitotic spindle. The *CIN1* gene of *S. cerevisiae* encodes a non-essential putative tubulin cofactor, believed to be involved in the polymerisation of α– and β– tubulin dimers (Tian, Huang et al. 1996; Hoyt, Macke et al. 1997). Consequently, this makes cells more susceptible to perturbations in spindle assembly. A *cin1Δ* strain only becomes compromised when an additional agitatant is enforced upon the mitotic spindle, such as the addition of the microtubule antagonist, benomyl.

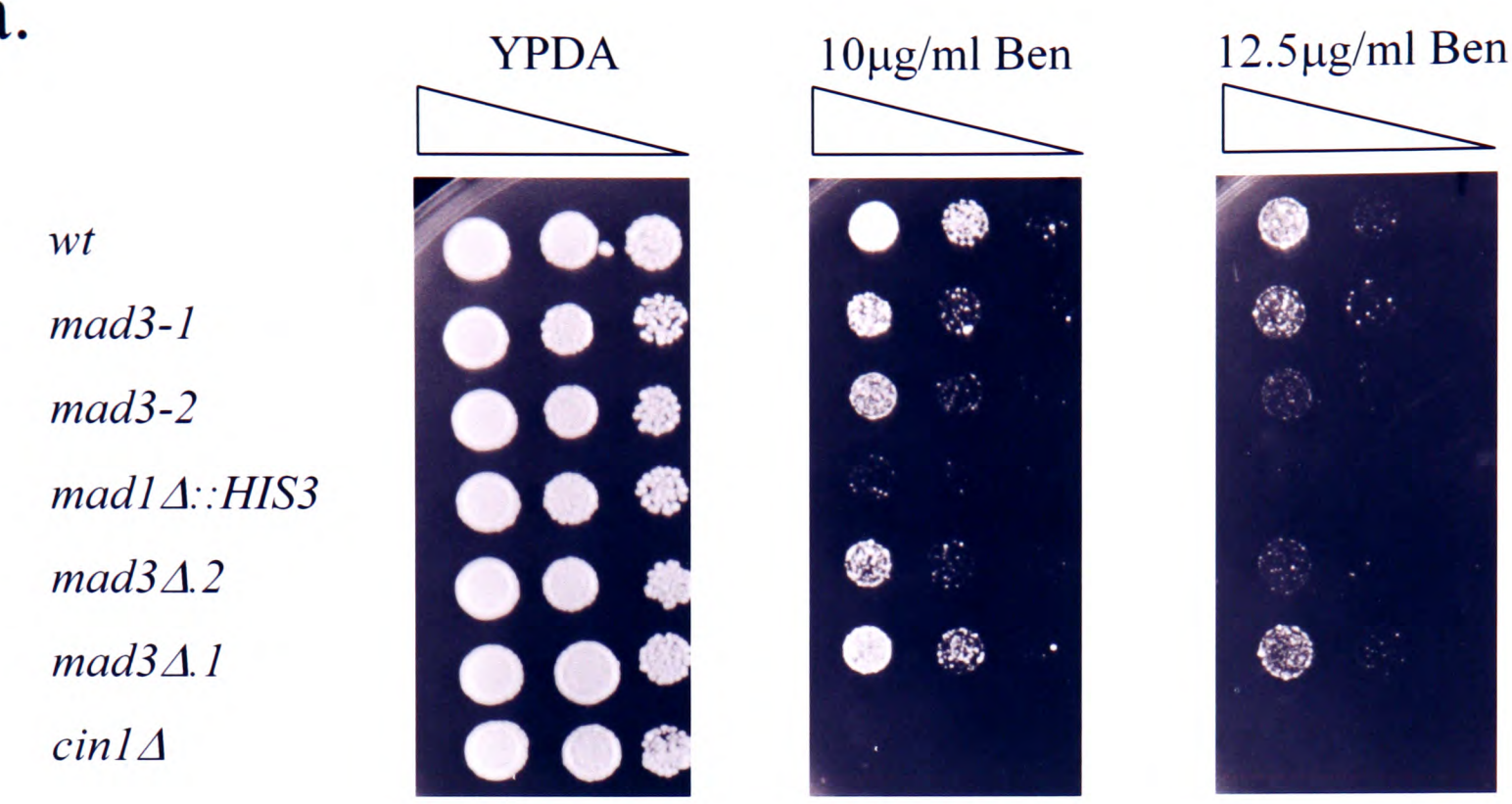
The *mad3* mutants were indeed benomyl sensitive similar in manner to the *mad1* mutant and strikingly different to the sensitivity displayed by the *cin1* mutant (Fig.3.2a). However, the *mad3* sensitivity was not the same as that found for the *mad1* mutant suggesting different levels of importance for Mad3p and Mad1p within the spindle checkpoint machinery.

3.2.4 *mad3* mutants divide faster than wild type cells on benomyl plates

As discussed at the beginning of this chapter a spindle checkpoint mutant must comply with two criteria: a mutant allele of the component must be sensitive to perturbations in the mitotic spindle and, it must divide faster than a wild type strain on benomyl plates. Currently the best way to show this increased rate of division is to perform a microcolony assay with the checkpoint mutant and compare this with the division rate of a wild type strain as illustrated in Figure 1.5 and described in section 2.2.1.10. To test the rate of division of *mad3* mutants on benomyl plates the *mad3Δ 1* strain was grown on YPDA plates containing 12.5μg/ml benomyl over a period of 12 hours. Figure 3.2b clearly shows that *mad3* mutants divide faster than wild type cells under these conditions, dividing at a rate approaching that of the *mad1* mutant. The difference between a checkpoint mutant and a *cin1Δ* is apparent in this experiment. The *cin1Δ* buds formed two cells but never continues to divide due to the total absence of a mitotic spindle (Fig 3.2b). The initial rapid division of the *mad* mutants compared to wild type eventually led to their death and the continued growth of the wild type strain as evident in figure 3.2a. This assay was performed for over 100 cells for each strain analysed and repeated at least three times.

Figure. 3.2

a.



b.

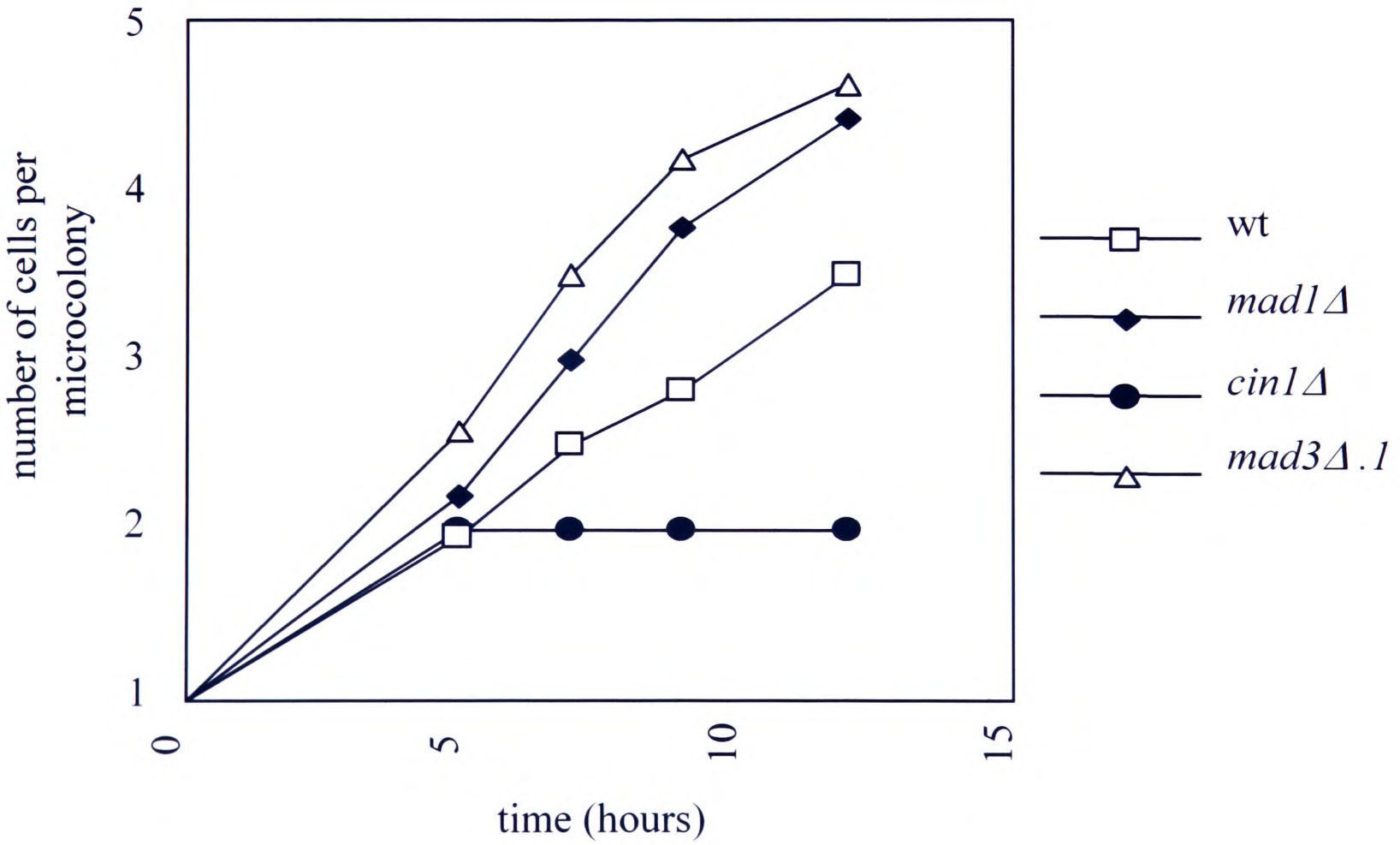


Figure 3.2 – A) *mad3* mutants are benomyl senstive, a 10 fold dilution of cells from left to right is indicated. B) *mad3* mutants divide faster in the presence of benomyl. These data are consistent with previously identified components of the spindle checkpoint, *mad1Δ*

3.3 Mad3p is a ubiquitously expressed nuclear protein

3.3.1 Affinity purification of anti-Mad3p antibody

Rabbit serum containing Mad3-GST (glutathione S-transferase) antibodies was produced in San Francisco by injecting a rabbit with a Mad3-GST fusion protein and collecting blood at monthly intervals for 3 months. At the end of this time the rabbit was exanguinated. The serum obtained was passed over an Affigel 10 column with covalently bound GST. This was repeated once and the flow through was passed over a protein column containing covalently bound Mad3-GST fusion protein. This column was washed with buffer and eluted with low pH Glycine. The eluate was dialysed into PBS containing 0.02% azide. Figure 3.3(a) shows Mad3p at approximately 60KDa.

3.3.2 Production and affinity purification of anti-Bub3p antibody

A high copy number bacterial plasmid containing the *BUB3* open reading frame fused to GST was a kind gift from A. Hoyt. The Bub3-GST fusion was expressed from the IPTG inducible reporter. The fusion protein was injected into a rabbit by Diagnostics Scotland (Ellen's Glen Road, Edinburgh, EH17 7QT) and serum was collected at monthly intervals for 3 months at which point the animal was exsanguinated. The anti-Bub3p antibody was purified as previously described for the anti-Mad3p antibody (Section 3.2.1). Figure 3.3(b) shows the specificity of the antibody, recognising a protein of approximately 25KDa present in a *wt* strain but absent from a *bub3Δ* strain. A more intense Bub3p signal was detected in a *wt* strain expressing *BUB3* from a 2μ plasmid using the *GAL1* promoter; this plasmid was a kind gift from Frank Solomon (Guenette, Magendantz et al. 1995).

This antibody was used to visualise Bub3p by Brady and Hardwick (Brady and Hardwick 2000) but its short lifespan (<month) prevented its use in this study. All subsequent Bub3p immunological work was performed using a *BUB3-myc*$_{13}$ strain

Figure 3.3

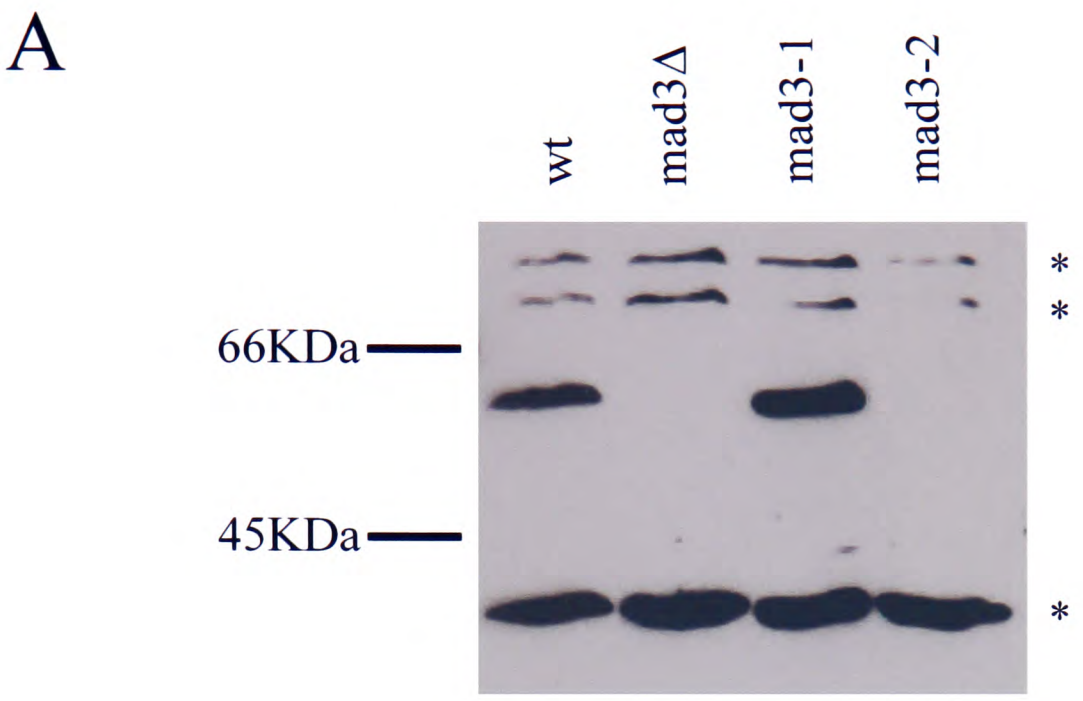


Figure 3.3 - A) The affinity purified anti-Mad3p antibody recognises a band running at approximately 60KDa which is absent from a *mad3Δ* strain and a strain known to contain a STOP codon at amino acid 83, *mad3-2*. * denotes background bands in *S. cerevisiae*. B) The anti-Bub3p antibody recognises a band running at approximately 25KDa in a wild type strain, wt, and a strain expressing *BUB3* from the GAL4 promoter on a 2μ plasmid.

constructed in A. Murray's laboratory, KH242. Bub3p was detected using an anti-MYC epitope antibody (Table 2.1.10).

3.3.3 Mad3p levels are constant throughout the cell cycle

A *wt* strain was grown to log phase in YPDA. This log phase culture was then arrested using 10μg/ml α-factor until >90% of the population produced mating projections; a result of the cells arresting in G1 as they prepare to mate. This 100ml culture was then centrifuged at 3000rpm for 3min to remove the α-factor, and resuspended in 100ml YPDA. It was then split into two 50ml cultures from which a 5ml sample was taken for the zero time point. One culture was arrested with 15μg/ml nocodazole and both were grown at 24°C. 5ml samples were taken at 15min intervals. Western blot analysis was performed on the samples (Figure 3.4). Figure 3.4 shows that Mad3p levels remain constant throughout the cell cycle and no modifications are visible during metaphase (high Clb2p levels) or nocodazole treated extracts. The cultures were synchronised in G1 efficiently with α-factor and continued through mitosis or arrested, as shown by sustained Clb2p levels; the major B-type cyclin in *Saccharomyces cerevisiae*.

3.3.4 Mad3p is a nuclear protein

Cultures were grown to log phase in YPDA or selective media. The cells were treated as described for immunofluorescence (Section 2.2.2.2). *wt* and *mad3Δ* strains were incubated in anti-Mad3p antibody at a range of dilutions and stained with DAPI to visualise the DNA. Unfortunately no signal was detected using the antibody (data not shown) possibly due to the low expression levels of Mad3p or the inability of the antibody to recognise native Mad3p. To address this problem Mad3p localisation was assayed in cells over-expressing *MAD3*.

A 2μ plasmid containing *MAD3* being expressed from the TPI promoter (pKH513) was transformed into *wt* cells. This culture was treated as described for immunofluorescence microscopy in section 2.2.2.2. The 2μ vector propagates randomly in a population resulting in a range of 1 to 100+ copies per cell (Van der

Figure 3.4

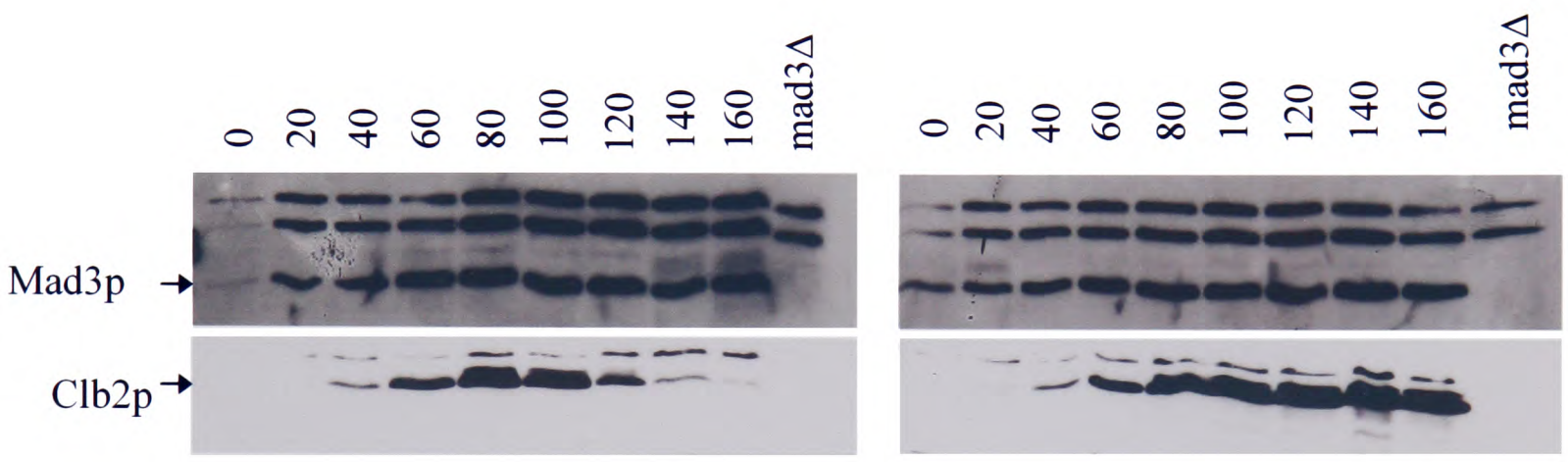


Figure 3.4 - Mad3p levels are not cell cycle regulated. Both sets of samples are from a culture synchronised in G1 using an α-factor arrest. The protein levels of Clb2p rise as the cells enter mitosis reaching a peak at 80-100mins. The levels then decrease in the absence of nocodazole to G1 levels. In nocodazole treated cells the levels of Clb2p remain high as the cells arrest in metaphase. Mad3p levels do not change throughout the cell cycle of *S. cerevisiae*. The low level of Mad3p in the 0mins time sample reflect an overall low protein loading in this sample. No Mad3p is detected in the *mad3Δ* sample.

Sand, Greenhalf et al. 1995). This results in a gradient of staining for Mad3p in a broad field of cells (Fig. 3.5(a)). The fact that Mad3p is seen in the nucleus at all detectable levels of expression suggests that at *wt* levels it is also present in the nucleus.

It is possible to observe Mad3p outside the DAPI signal suggesting that Mad3p may not always be contained within the nucleus (Fig. 3.5(a)). To confirm that the Mad3p was always found within the nucleus an antibody against a known lumenal endoplasmic reticulum (ER) protein, Kar2p, was used. The nuclear membrane is contiguous with the ER in *S. cerevisiae* allowing the localisation of Kar2p to outline the nucleus (Latterich and Schekman 1994). Mad3p localisation was restricted to inside the ring detected by the anti-Kar2p antibody (Fig. 3.5).

3.4 Discussion

The work presented in this chapter provides evidence that the *mad3-1* and *mad3-2* strains encode alleles of the YJR013c gene open reading frame. 3.2.1 shows a linkage between the YJR013c ORF and the locus conferring benomyl sensitivity upon the *mad3-1* and *mad3-2* strains. Sequencing of this ORF in both strains revealed point mutations in each.

Previous work using *mad3* mutants strengthens the data presented in this chapter. *mad3* mutants have been shown to behave in a similar manner to a known component of the checkpoint in sister separation experiments (Straight, Belmont et al. 1996). These experiments monitor the premature separation of sister chromatids in the presence of another microtubule depolymerising agent similar to benomyl, namely nocodazole. Wild type cells delay the separation of sister chromatids in this assay but mutants within the mitotic checkpoint do not. A *mad3* mutant was found to separate sister chromatids at the same time as *mad1*, *mad2*, *bub1*, *bub2* and *bub3* mutants further strengthening the evidence for inclusion of Mad3p in the mitotic checkpoint. This paper also monitored the rate of death of *mad* and *bub* strains in the presence of nocodazole. Mutants in the checkpoint components, including *mad3Δ*, died rapidly in the nocodazole whereas wild type cells did not and cells containing a *tub1* mutant died after a long delay corresponding to the approximate length of a budding yeast cell

Figure 3.5

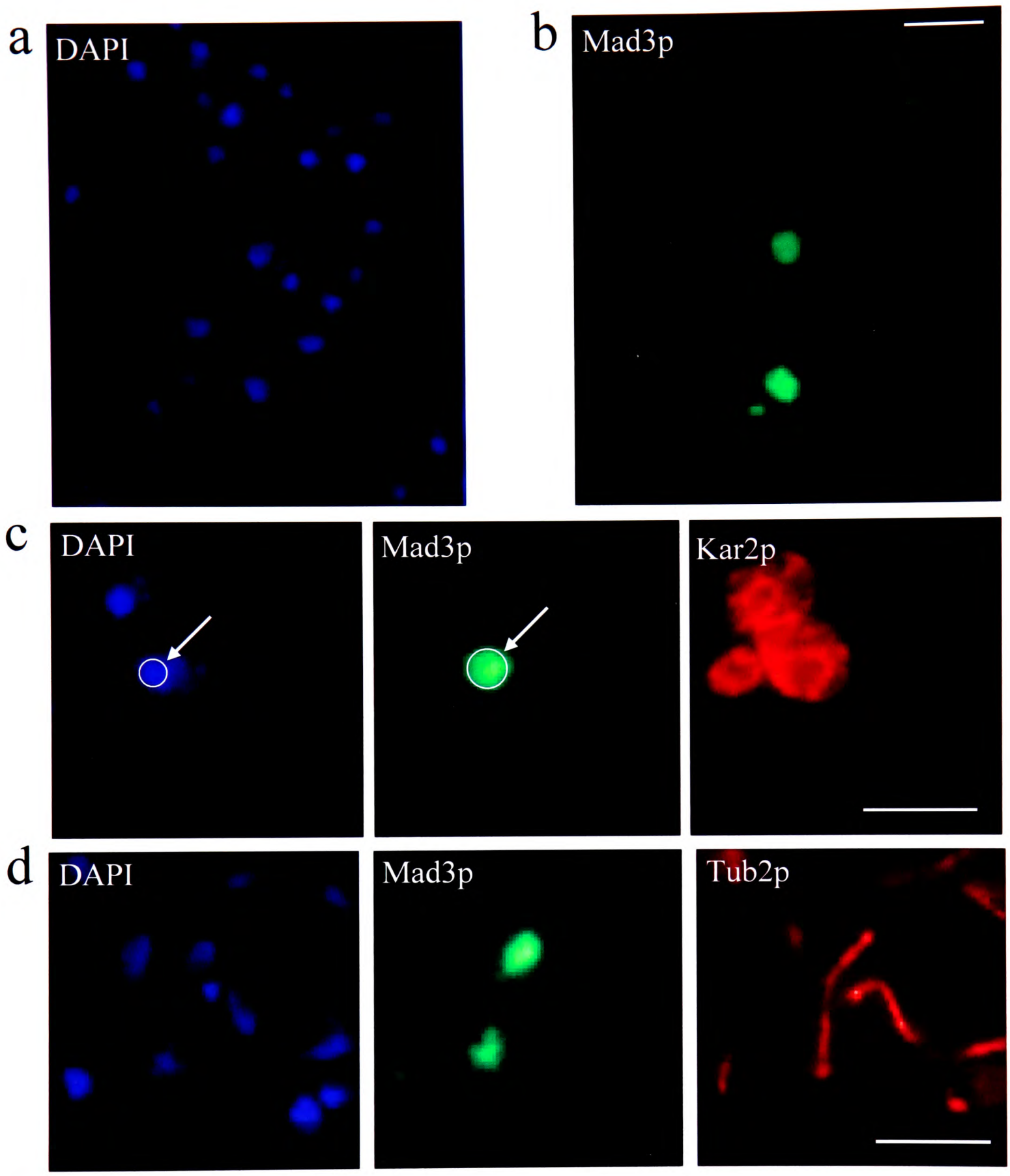


Figure 3.5 - Mad3p is present in the nucleus when over-expressed. (a) and (b) Broadfield image of asynchronous culture, (c) Co-staining with anti-Mad3p and anti-Kar2p antibodies showing Mad3p localisation is restricted to the nucleus, (d) Co-staining with anti-Mad3p and anti-Tub2p antibodies showing normal tubulin localisation in *MAD3* over-expressing cells. The bar represents 10μm

cycle. Thus adding credence to the argument for inclusion of Mad3p in the mitotic checkpoint (Straight, Belmont et al. 1996).

Further evidence using the over-expression of the *MPS1* protein kinase also supports a checkpoint role for the gene product of YJR013c (Hardwick, Weiss et al. 1996). The over-expression of the *MPS1* protein kinase from the *GAL1* promoter arrests cells in mitosis in a way reminiscent of checkpoint activation. Over-expression of *MPS1* in a *mad3* mutant strain does not result in arrest suggesting that Mad3p acts downstream from Mps1p. Additionally, Mad1p was found to be phosphorylated in *mad3Δ* cells containing *GAL-MPS1* suggesting Mad3p also acts downstream of Mad1p phosphorylation. The arrest in *MPS1* over-expression cells was also lost *mad1*, *mad2*, *bub1*, *bub2* and *bub3* strains (Hardwick, Weiss et al. 1996).

Mad3p is present at constant levels throughout the cell cycle of *S. cerevisiae* and does not appear to be modified at any point even when the checkpoint is active as evident in the nocodazole treated extract where Clb2p is stabilised. This is not true for the mammalian homologue of Mad3p, BubR1, which is indeed phosphorylated and displays a cell cycle dependent localisation pattern (Taylor, Hussein et al. 2001). The localisation of Mad3p may be altered when the checkpoint is activated. Unfortunately, the protein could not be detected in *wt* cells, preventing analysis of its localisation throughout the cell cycle. However, when over-expressed, Mad3p was only visible in the nucleus in a log phase culture so it is clear that it is present in the nucleus (Figure 3.5).

Many sub-cellular components are easily visualised in the fission yeast *Schizosaccharomyces pombe*. Work by a colleague in the laboratory has localised *S. pombe* Mad3 to the kinetochore of fission yeast (Millband and Hardwick 2002). Work in mammalian cells shows specific localisation of human BubR1 to kinetochores using light microscopy and electron microscopy (Jablonski, Chan et al. 1998; Taylor, Hussein et al. 2001). This suggests that the localisation of Mad3p is indeed important for its checkpoint activity in higher eukaryotes.

An alternative strategy to immuno-localisation has been employed by the Spencer group to detect Mad3p at the kinetochore of budding yeast. The 1-hybrid assay allows the detection of proteins which interact with specific regions of DNA, including the centromere of *S. cerevisiae* (Ortiz, Stemmann et al. 1999). Using this

assay Mad3p was shown to interact with the kinetochore (C. Warren; per com). Unfortunately this assay cannot be employed to follow the kinetochore/Mad3p association during a single cell cycle. It is possible to immunoprecipitate Mad3p from yeast using the antibody produced in this chapter and once immunoprecipitated PCR could be employed to detect centromere specific sequences. This method, known as CHIP (CHromosome Immuno-Precipitation) analysis, may provide an insight into the localisation of Mad3p within the kinetochore complex during the cell cycle. Preliminary work using CHIP in this laboratory has localised Mad3p to the central region of the kinetochore illustrated in figure 1.3 (K. Hardwick, per comm.).

Chapter 4 - Mad3p interacts with Cdc20p and Bub3p via two distinct domains

4.1 Mad3p shares two domains of homology with Bub1p

The previous work in this study confirms Mad3p as a component of the spindle checkpoint. The next step is to offer some role for Mad3p within the checkpoint machinery.

A BLAST search of the *Saccharomyces cerevisiae* genome scored the NH_2-terminus of Bub1p as sharing a high degree of homology with Mad3p (Fig. 4.1). The C-terminus of Bub1p contains a kinase domain absent from Mad3p.

A BLAST search of other organisms identified a *MAD3* homologue in *S. pombe* with no kinase domain. However, the mammalian homologues of Bub1 and Mad3 all contain a kinase domain (Figure 4.2). Indeed hBubR1 was originally identified as a *BUB1* homologue due to the presence of a kinase domain (Taylor, Ha et al. 1998). Closer inspection of hBubR1 identified an N-terminal extension absent from Bub1 in other species yet present in Mad3p of *S. cerevisiae* (Figure 4.2). A KEN box is located in this N-terminal extension (Figure 4.3). This Lysine-Glutamate-Asparagine motif has been implicated in targeting APC/Cdh1p to specific targets in mammalian cells (Pfleger and Kirschner 2000) and was recently identified as targeting the APC dependent degradation of Cdc20p and Cdh1p in yeast (Burton and Solomon 2001).

Two-hybrid analysis performed in A. Murray's laboratory using known components of the spindle checkpoint revealed interactions between Mad3p and Bub1p, and Cdc20p and Bub3p (Hardwick, Johnston et al. 2000). It is important to note from the two-hybrid data that Bub1p shows a strong interaction with Cdc20p, however, Cdc20p was not detected in an immunoprecipitation of Bub1p (Brady and Hardwick 2000). The physical interaction between Bub1p and Bub3p supported a genetic interaction between the two proteins shown in a genetic screen for high copy number suppressors of a *bub1* mutant (Hoyt, Totis et al. 1991).

Further reduction of Mad3p in this two-hybrid study identified the first region of homology being sufficient for the interaction with Cdc20p and the second region of homology being sufficient for the interaction with Bub3p (Hardwick, Johnston et al. 2000). To confirm these interactions the Murray laboratory added Mad3p-GST to

Figure 4.1

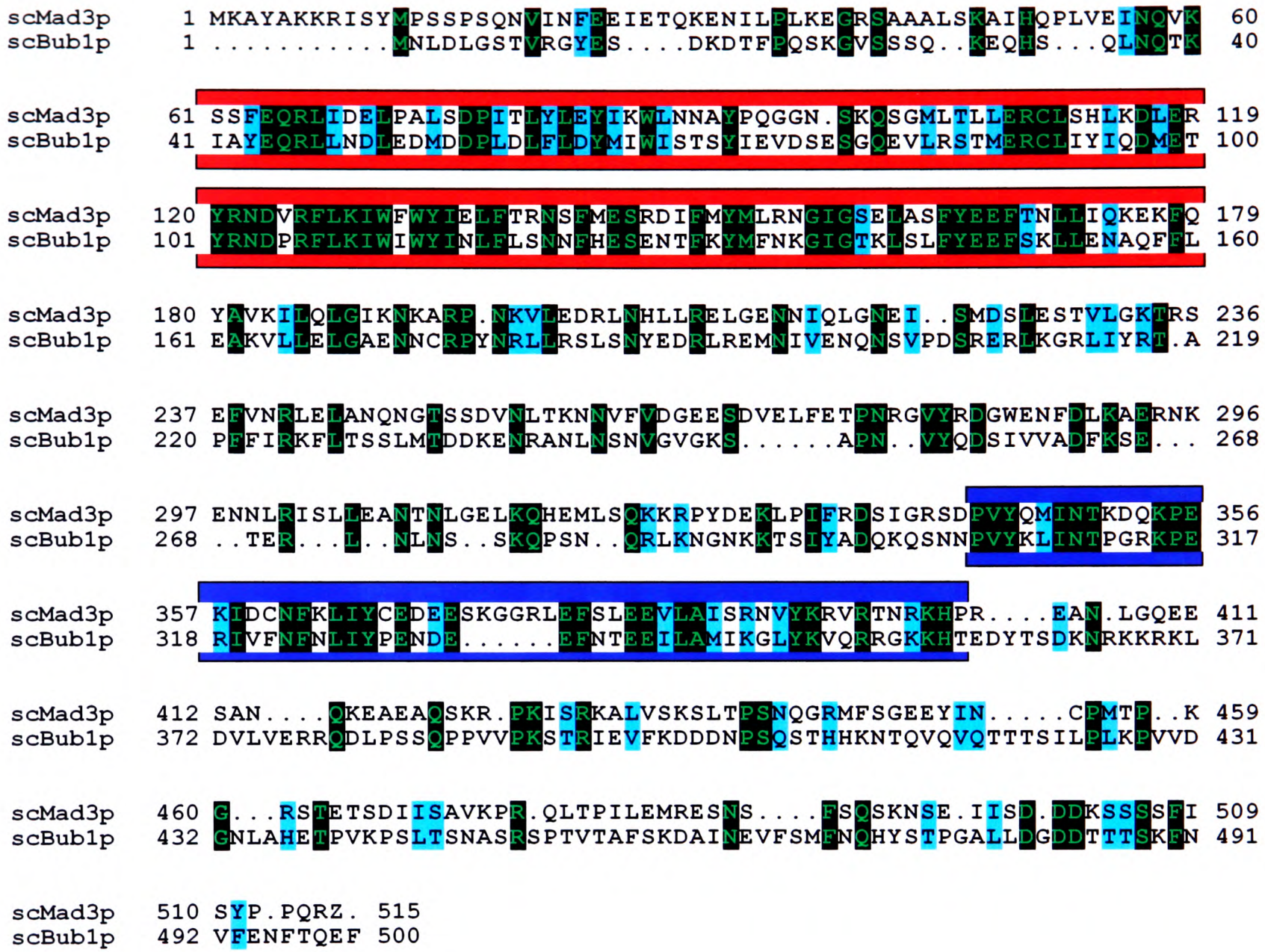


Figure 4.1 - Alignment of *Saccharomyces cerevisiae* Mad3p and the first 500 amino acids of Bub1p. Blue box indicates first stretch of high homology, region I. Red box indicates second stretch of high homology, region II. The alignment was performed using the Clustal X program. The alignment was then coloured according to degree of homology using MacBoxShade.

Figure 4.2

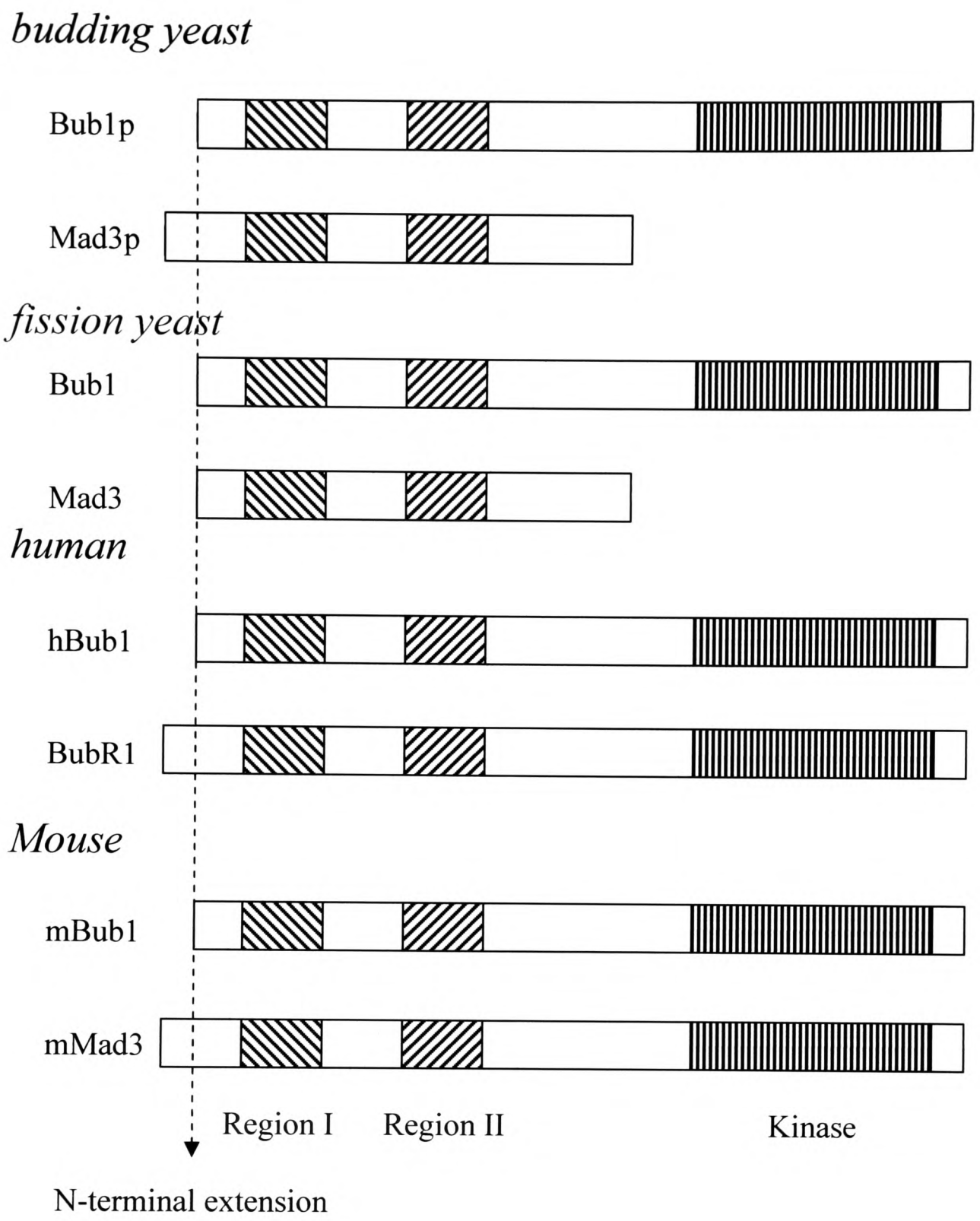


Figure 4.2 - The structure of Mad3 and Bub1 homologues. The kinase domain is absent from yeast Mad3. The NH_2-terminal extension is found in all Mad3p homologues.

Figure 4.3

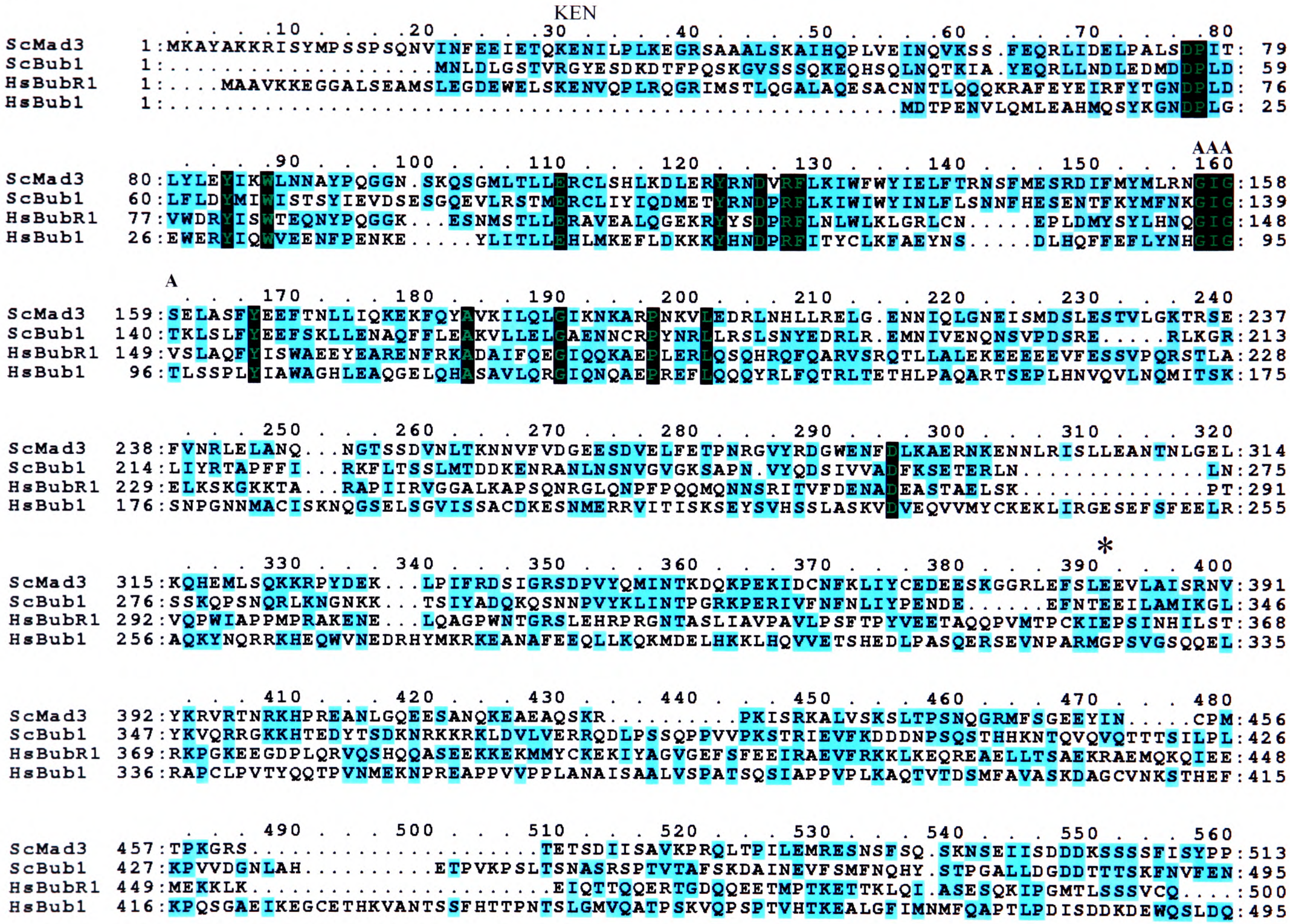


Figure 4.3 - Alignment of Mad3 and Bub1 from yeast and humans shows conserved residues. The region I mutant is marked AAAA. The region II mutant (mad3-1) is marked "*". The KEN boxes of ScMad3p and HsBubR1 are marked by "KEN".

rabbit reticulocyte lysates containing radioactive Bub3p or Cdc20p (Hardwick, Johnston et al. 2000). Bub3p and Mad3p-GST were shown to interact in this assay however the Cdc20p and Mad3p-GST interaction could not be detected at levels above Cdc20p and GST alone. As there are no other yeast proteins present in these lysates the Bub3p and Mad3p-GST interaction is likely to be independent of other proteins. Perhaps the Mad3p and Cdc20p interaction may require additional proteins or modifications not possible in the *in vitro* assay.

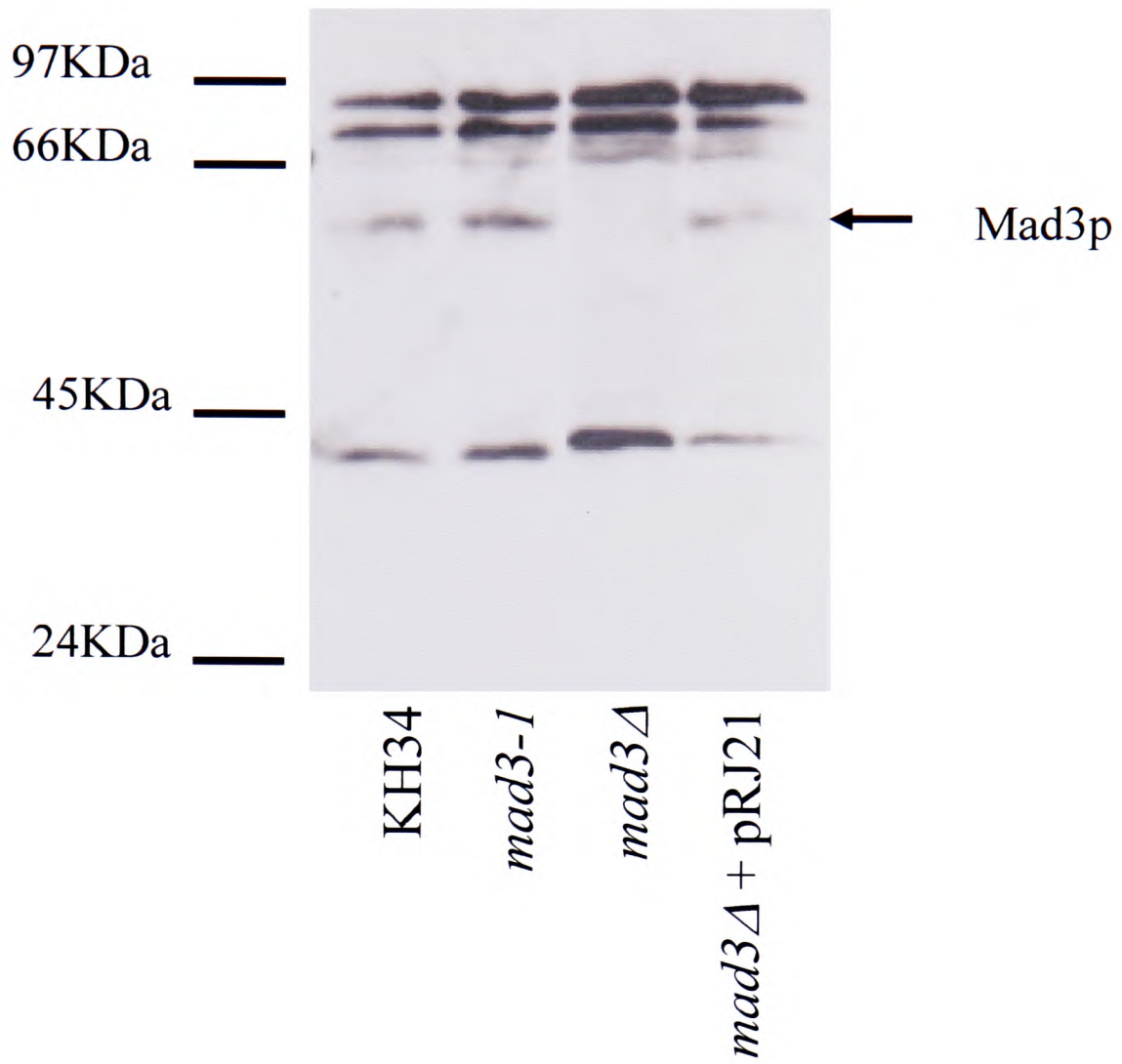
The two-hybrid and *in vitro* reticulocyte lysate studies strongly suggest that the N-terminus of Mad3p interacts with Cdc20p and the C-terminus of Mad3p interacts with Bub3p. This chapter aims to prove the interactions *in vivo*.

4.2 Region I of Mad3p is required for Mad3p – Cdc20p association *in vivo*

The GIGS motif found in Mad3p shows a high degree of homology across the Mad3p and Bub1p homologues (Figure 4.3). This motif was replaced with four alanines introducing a NotI restriction site in the DNA using the primers, 3T1 and 3T2. 500bp of the 5' non-coding region of *MAD3* was inserted into a pRS316 vector. The PCR products of 3.30 – 3T1 and 3T2 – 3.31 (Table 2.1.8) were then ligated to the non-coding region in the pRS316 vector producing the *TRP1* marked single copy plasmid encoding a *mad3* region I mutant, pRJ21. The benomyl sensitivity of this mutant was tested as was the expression level of the mutant protein (Figure 4.4). A triple Haemagglutinin (HA) tagged Cdc20p plasmid, pLH68, was transformed into strains and was detected using an anti-HA antibody (2.1.1.11). pLH68 was transformed into three strains; *wt* (KH34); *mad3Δ*, a Ura$^-$ derivative of KH173; *mad3-1* (KH 45). pRJ21 was also transformed into an additional *mad3Δ* strain containing pLH68. An anti-HA antibody bound to protein A beads was used to pull down Cdc20p^{3HA} protein and the precipitates were western blotted. Mad3p was detected using the polyclonal antibody (Table 2.1.10). This experiment was performed by K. Hardwick ((Hardwick, Johnston et al. 2000) Figure.4b which can be found in an

Figure 4.4

a)



b)

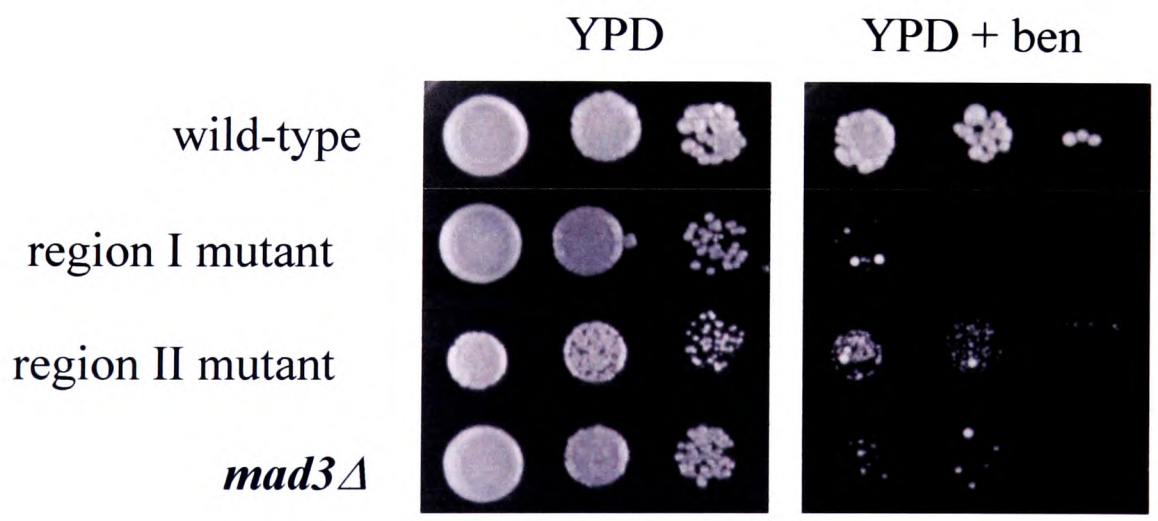


Figure 4.4 - The region I and II mutants are expressed in cells at wild type levels and are benomyl sensitive. a) pRJ21 expresses wt levels of the region I mutant protein. Antibodies were used as described in Figure 4.1. b) Both region I and region II are benomyl sensitive at levels comparable to a *mad3Δ* strain. 12.5 µg/ml of benomyl was used.

appendix to this thesis). A band corresponding to Mad3p was detected in the region II mutant and the wild type lanes which was absent form the lane containing the region I mutant. Mad3p protein was present in all precipitates with the exception of the *mad3Δ* lane.

4.3 Region II of Mad3p is necessary for Mad3p – Bub3p interaction *in vivo*

The *mad3-1* mutant isolated in a screen for benomyl sensitive strains in 1992 (K. Hardwick; per comm) when sequenced contained a point mutant (Section 3.2.2). Residue 382 was altered from a glutamate residue to lysine.

Strains containing the *BUB3* gene with 13 Myc epitopes tagged to the 3'-end of the gene were constructed. These strains are; YRJ12 (wt); YRJ 10 (*mad3Δ*); YRJ11 (*mad3-1*). pRJ21 was transformed into YRJ 10. The level of expression of the *mad3-1* mutant was testing and confirmed as being equivalent to wild type levels of the protein (Figure 4.4a). The benomyl sensitivity of the mutant was also tested and shown to be similar to the benomyl sensitivity of a *mad3Δ* (Figure 4.4b). Anti-Mad3p or anti-Myc antibody were used to immunoprecipitate either Mad3p or Bub3p from the five strains; KH 34; YRJ12; YRJ 10; YRJ 10 + pRJ21; YRJ 11. The immunoprecipitation protocol was followed as in materials and methods. Figure 4.5 shows the results of the immunoprecipitation. Bub3p-Myc_{13} co-immunoprecipitated with wild type Mad3p and the region I mutant but did not immunoprecipitate with the *mad3Δ* strain or the region II mutant (Figure 4.5a) In the reverse immunoprecipitation wild type Mad3p and the region I mutant both co-immunoprecipitated with Bub3p-Myc_{13} whereas no Mad3p was detected in the immunoprecipitates from the *mad3Δ* strain or the region II mutant (Figure 4.5b). In both cases no interaction was seen in the wild type strain containing untagged Bub3p nor in the *mad3Δ* strain showing that these results are not due to antibody cross-reactions.

Figure 4.5

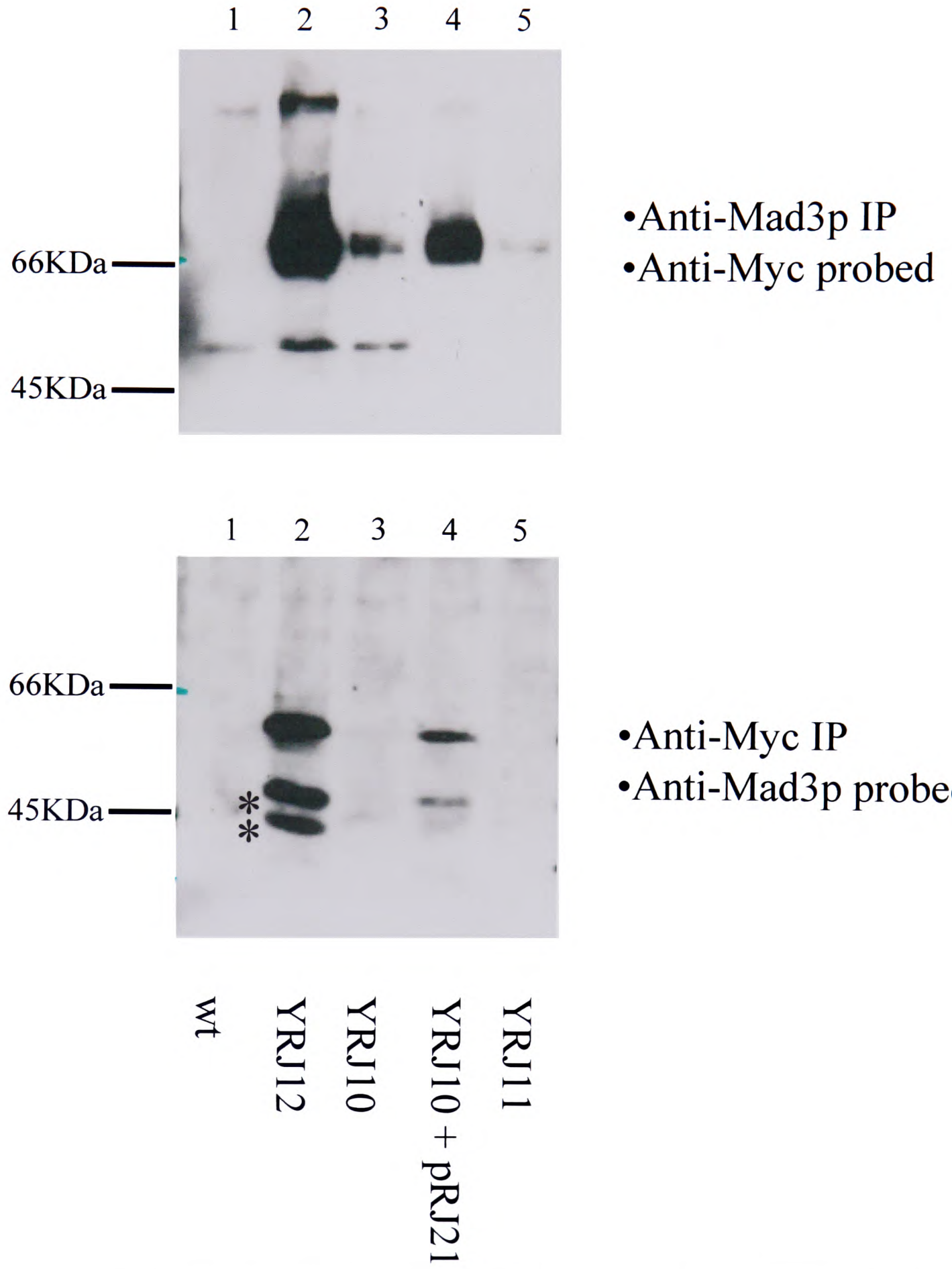


Figure 4.5 - Mad3p and Bub3p interact *in vivo*. A) Bub3-Myc3 precipitates with Mad3p (YRJ12) and the region I (YRJ10 + pRJ21) mutant but not the region II (YRJ11) mutant in tagged strains in a specific manner. No signal is detected in the wt lane which does not contain a tagged Bub3p. B) Bub3p precipitates with both Mad3p and region I mutant but not with region II mutant or *mad3Δ*. The two marked bands indicate degradation products of Mad3p.

4.4 Bub3p-myc13 associates with both Mad3p and Bub1p during G1, S, M and in nocodazole arrested cells.

Bub1p and Bub3p have been shown to interact in budding yeast and that amino acids 141-609 of Bub1p are sufficient for that interaction (Roberts, Farr et al. 1994). A mutation in the same region of Mad3p destroys the Mad3p and Bub3p interaction showing that this domain of Mad3p is necessary for the Mad3p/Bub3p interaction. Do the interactions between Mad3p/Bub3p and Bub1p/Bub3p form at different points in the cell cycle? To test this a strain containing a *cdc26Δ* and the Bub3p-myc$_{13}$ locus was constructed, YRJ13. *CDC26* encodes a non-essential component of the APC (Zachariae, Shin et al. 1996). A *cdc26Δ* strain arrests in mitosis when shifted to 37°C allowing the analysis of a synchronous population of yeast in mitosis without the use of nocodazole, leaving the mitotic checkpoint switched off (Hwang and Murray 1997).

YRJ13 was arrested in α-factor, hydroxyurea (HU), nocodazole or shifted to 37°C for 2 hours. A sample was checked under a microscope to ensure an arrest had occurred (data not shown). The cultures were then subjected to the immunoprecipitation protocol described earlier. Mad3p was found in all lanes showing that the Mad3p/Bub3p complex is not cell cycle regulated (Figure 4.6). Bub1p is also present in all lanes showing that the Bub1p/Bub3p complex is also not cell cycle regulated. However, the different species of Bub1p found in each lane illustrates the different phosphorylation states of Bub1p during the cell cycle. The maximum Bub1p bandshift seen in nocodazole arrested compared to α-factor and HU arrested cells suggests that Bub1p is phosphorylated in a mitotic checkpoint dependent manner. The low level of phosphorylation in the *cdc26Δ* strain is not surprising as the cells in this arrest are unlikely to have any mitotic abnormalities.

4.5 Discussion

The work in this chapter investigates the interactions between Mad3p and Bub3p and Mad3p and Cdc20p *in vivo*. And in so doing, not only confirm work shown

Figure 4.6

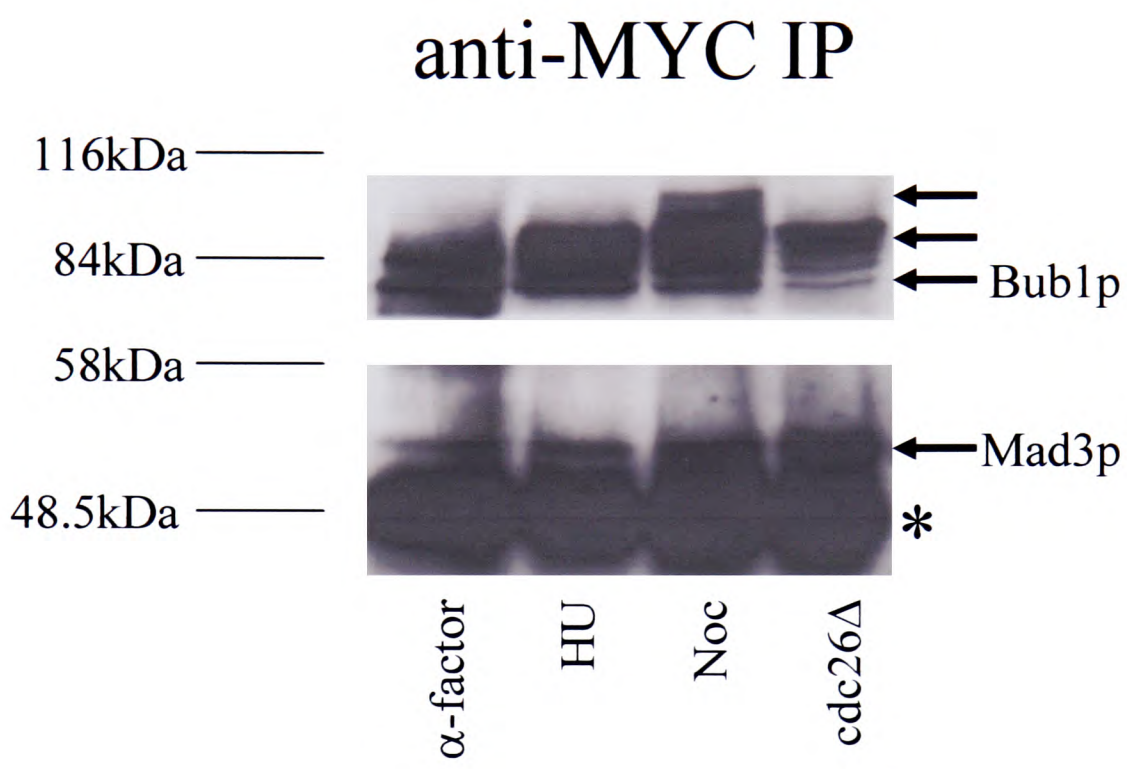


Figure 4.6 - Bub3-13MYCp was immunoprecipitated with the anti-MYC antibody and the precipitates were probed with either anti-Bub1p or anti-Mad3p. Both Bub3/Bub1p and Bub3/Mad3p interactions form at several parts of the cell cycle. Cells were arrested with α-factor, hydroxyurea (HU), nocodazole (Noc) or a temperature shift in a *cdc26Δ* strain (see text). Mad3p is indicated on the lower blot. Different species of phosphorylated Bub1p are indicated on the upper blot. * indicates the antibody light chain.

by two-hybrid and in vitro binding techniques, but also identify the regions responsible for those interactions.

The construction of a *mad3* mutant in the first region of homology to Bub1p ($GIGS_{159} \rightarrow AAAA$) was used to show the necessity of region I of Mad3p for the interaction with Cdc20p (Hardwick, Johnston et al. 2000). By using a *mad3* mutant which contained a mutation in the second region of homology to Bub1p (*mad3-1*) region II was shown to be necessary for the interaction between Mad3p and Bub3p (Figure 4.5). As these mutations confer benomyl sensitivity upon yeast strains the interactions have a functional significance within the mitotic checkpoint (Figure 4.4b).

Region II of both Mad3p and Bub1p have been shown to interact with Bub3p (this work and (Roberts, Farr et al. 1994) respectively) in yeast and humans (Taylor, Ha et al. 1998) despite low homology in this region of the two proteins across the species (Figure 4.3). The function of this domain in mammalian cells is to localise Bub1 and BubR1 to the kinetochore of sister chromatids which are unattached to the mitotic spindle (Taylor, Ha et al. 1998). In yeast it has not been possible to localise Mad3p or Bub1p more specifically than to the nucleus (Section 3.3.4 and (Roberts, Farr et al. 1994) respectively) but this may be the purpose of the Bub3p interactions of these two proteins. This hypothesis suggests the formation of these complexes to be cell cycle regulated, only forming when the checkpoint is active or early in mitosis of living cells, however, figure 4.6 shows the binding of Mad3p to Bub3p is not cell cycle regulated but persists through G1, S and unperturbed mitotic cells. The Mad3p - Bub3p interaction was also shown to be independent of other mitotic checkpoint components (Hardwick, Johnston et al. 2000) a result obtained for the Bub1p - Bub3p interaction by other members of the Hardwick laboratory (Brady and Hardwick 2000). Figure 4.6 shows this Bub1 - Bub3p complex also persists throughout the cell cycle. There are now three constitutive complexes within the mitotic checkpoint; Mad3p/Bub3p, Bub1p/Bub3p and Mad1p/Mad2p (Chen, Brady et al. 1999). Mutations disrupting these complexes make cells benomyl sensitive confirming their importance in the functioning of the checkpoint.

Region I of Mad3p is responsible for the interaction between Mad3p and Cdc20p in yeast (Hardwick, Johnston et al. 2000). This is an interesting result as Cdc20p is the target of the mitotic checkpoint. By inhibiting the activity of Cdc20p the

APC can be prevented from prematurely catalysing the separation of the sister chromatids.

Mad2p was also found in the immunoprecipitates of Mad3p and Cdc20p, and was shown to be required for the formation of the Mad3p - Cdc20p interaction (Hardwick, Johnston et al. 2000). Despite the high degree of homology in region I of Bub1p and Mad3p, Bub1p was not shown to immunoprecipitate with Cdc20p during any point in the cell cycle (data not shown). However, Bub1p has been reported to bind to Mad1p upon mitotic checkpoint activation and Mad2p is necessary for this interaction. Could the function of region I be to bind to the interface of Mad2p and another protein with subtle nuances in the sequence allowing the specific interaction of Mad3p and Cdc20p and Bub1p with Mad1p? Could this be the function of the NH_2-terminal extension of the Mad3p homologues? A way to test this hypothesis would be to transfer the NH_2-terminal extension onto Bub1p; would this chimera then bind to Mad2p/Cdc20 complex? This is a possible experiment for future workers.

Chapter 5 - A Screen for Mad3p Interacting Proteins Using the Two Hybrid System

5.1 Introduction

The previous chapters have shown an interaction between Mad3p and Bub3p and between Mad3p and Cdc20p. The *in vitro* experiments showed an interaction between Mad3p and Bub3p did not require other yeast proteins suggesting that the interaction between Mad3p and Bub3p does not require other proteins (Chapter 4 and (Hardwick, Johnston et al. 2000)). The interaction between Mad3p and Cdc20p, however, was dependent upon Mad2p *in vivo* yet no direct binding of Mad3p and Mad2p has been shown.

It is possible that the interaction between Mad3p and Cdc20p requires the presence of other proteins as well as Mad2p. It could be that the build up of mitotic proteins such as kinetochore components or microtubule associated proteins encourages the formation of a complex of Mad3p, Mad2p and Cdc20p to prevent anaphase during early mitosis.

5.2 Introduction to the Yeast Two-Hybrid System.

The two-hybrid system is a yeast-based genetic assay for detecting protein-protein interactions ((Fields and Song 1989); (Chien, Bartel et al. 1991); (Fromont-Racine, Rain et al. 1997); (Brent and Finley 1997) and references therein).

The assay relies upon the modular nature of transcriptional activator proteins, which are composed of two domains, a DNA binding domain (DNA BD) and an activation domain (AD); (Brent and Ptashne 1985). The DNA BD localises the activation domain to the promoter region, however, these two domains need not be covalently linked in order to function, since activity can be reconstituted through the interaction of any two proteins (Fields and Song 1989). The application of the assay requires the construction of two chimeric proteins *i*) the "bait" protein consisting of the DNA BD fused to the protein of interest (X) and *ii*) the "prey" protein consisting of the AD fused to the protein (Y) for which the interaction with X is being investigated. These two chimeric proteins are expressed in cells containing one or

more reporter genes. This relies upon the functional reconstitution of the transcriptional activator (and therefore interaction between X and Y) for their expression (see Figure 5.1).

The two-hybrid system can be used to assay directly if two proteins are capable of interacting, or alternatively, by creating a library of prey fusions, screens can be performed to identify novel interactions with the bait protein.

The two-hybrid system also has a number of advantages over classical genetic procedures for identifying and characterising protein-protein interactions. The main advantage is the ability to study interactions for which no conditional mutants are available. Other approaches rely upon mutant phenotypes to select for suppresser or enhancer mutations in genes whose protein products interact, however, in the two-hybrid assay the wild-type sequence can be used. This allows the interaction of proteins encoded by non-essential ORFs, or novel proteins for which conditional mutants are not yet available to be studied. Similarly, the system can be used to identify interactions with prey proteins encoded by non-essential ORFs. Thus, the two-hybrid assay can identify protein-protein interactions which would be missed by traditional genetic screens.

Furthermore, the availability of the *S. cerevisiae* genome sequence (Cherry, Ball et al. 1997) and SGD database) now allows all interacting factors isolated in screens to be identified.

Some bait fusions are capable of activating transcription of these genes even in the absence of prey fusions. However, by using the *HIS3* reporter gene any problems of such "auto-activation" can be alleviated by adding the competitive inhibitor, 3-aminotriazole (3-AT). This chemical is added to the medium used, to a level that makes the strain auxotrophic for histidine (even in the presence of the auto-activating bait) thereby allowing the interactions of that bait protein to be studied.

In this study, two-hybrid screens were performed using the mating strategy, FRYL library and prey analysis described by Fromont-Racine *et al.* (1997). Modifications of the basic two-hybrid assay by these authors allow rapid and

Figure 5.1

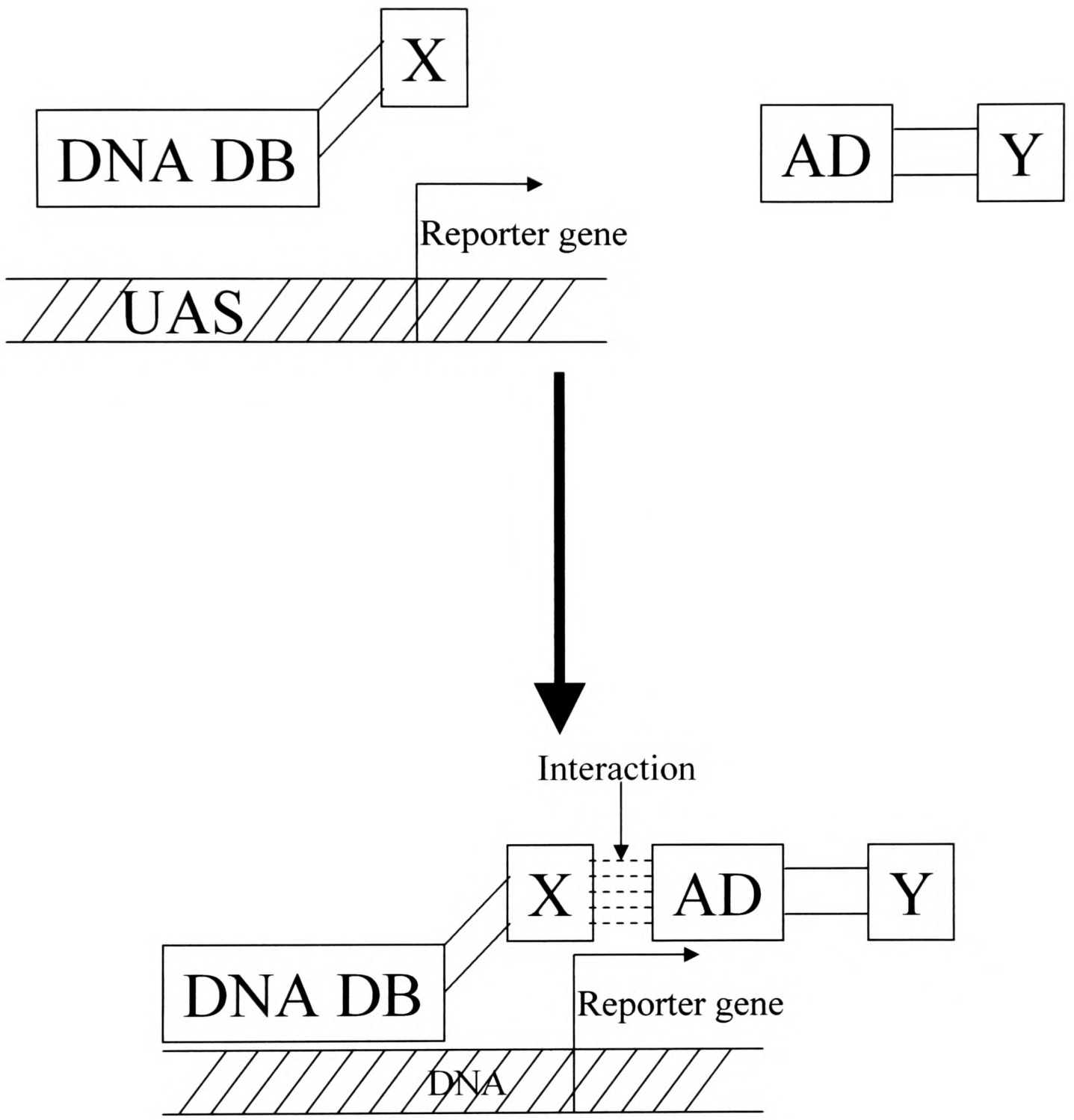


Figure 5.1 - The Two Hybrid system. UAS, upstream activating sequence. The two proteins of interest, protein X and protein Y, are co-expressed with the DNA binding domain (DNA BD) or the activation domain (AD) of a transcriptional activator protein. If an interaction occurs between protein X and protein Y DNA binding domain, which is bound to UAS sequence, can associate with the activation domain and transcription of the reporter gene occurs.

exhaustive screens of a yeast genomic library to be performed. The main advantages arise from the quality of the library and the way in which the results are analysed.

The FRYL library comprises $5x10^6$ clones, with randomly generated genomic inserts (average size 700bp), and a fusion event predicted to occur (statistically) every four bases throughout the genome. This will produce an in-frame fusion on average every 24 bases throughout the genome, however, Fromont-Racine *et al.* (1997) demonstrated that it is possible to select for and produce frame-shifted proteins from "out of frame fusions". The size of the library, and the frequency of fusion events, allows a greater number of potential interactions to be screened, and one wold expect to find a given ORF several times as independent clones. However, the probablility that a given fusion will be selected depends upon both the length of the interaction domain and on the position of that interacting domain along the coding sequence.

From these considerations, all prey were classified into one of five categories (Figure 5.2, as defined by Fromont-Racine *et al.* (1997)). The four "A" categories correspond to fusions beginning within the sequence of an ORF, while the "B" category relates to fusions located in an intergenic region; on the anti-sense strand of an ORF, in a non-protein encoding region (rDNA, telomeric DNA, mitochondrial DNA) or in a Ty retrotransposon element. The A1 category represents the most statistically significant interactions, with several fusions found as overlapping clones within a single ORF. As such, A1 prey share a common region which may be used to aid the identification of the interaction domain within the protein. The three other A categories represent candidates found only as a single fusion within an ORF, even if the same fragment is found several times. The A2 category consists of fusions starting close to the initiating codon of an ORF, and within 150 bases of the in-frame stop codon located upstream of this ORF. These fusions therefore represent amino-terminal interacting domains, and are expected to be under-represented in the library due to the presence of the in-frame non-sense codons upstream of the ORF which will interrupt translation. The A3 category represents candidates containing large coding inserts (greater than 1000bp) and may represent prey with a large interacting domain. This category will also be under represented since the average size of library inserts is 700bp. The A4 category represents all candidates in a coding sequence which do not fall into one of the other categories.

Figure 5.2

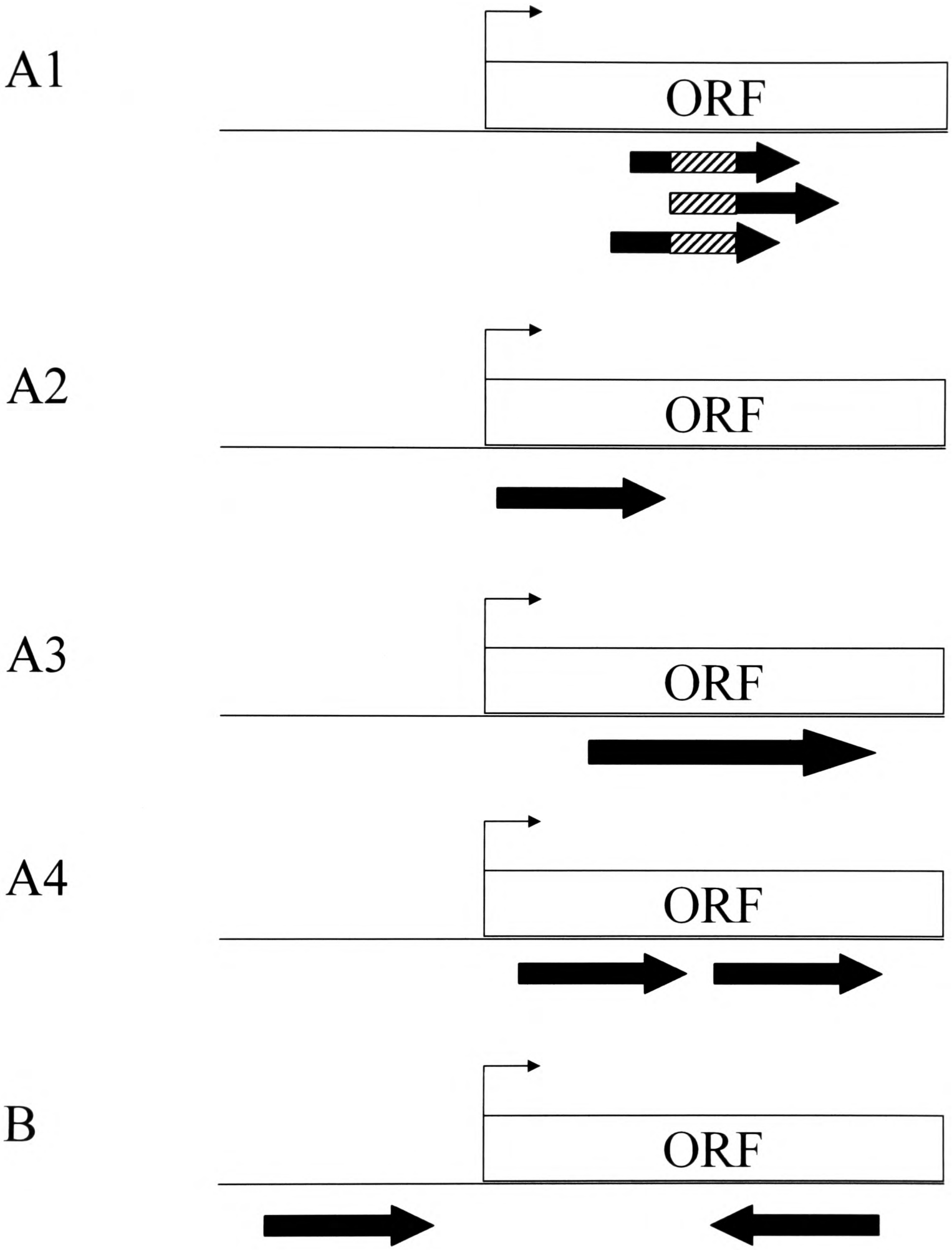


Figure 5.2 - The different categories of positives. These are graded in decreasing statistical significance, A1 to A4, or positves unlikely to have functional relevance.

The classification of prey found in a screen is only possible due to the reproducible experimental protocol which allows complete coverage of the library; $15x10^6$ interactions must be screened however the mating strategy typically allows exhaustive screens to be performed, with 20-60 million interactions tested.

5.3 Pre-screen Checks of the Gal4-Mad3p Fusion Protein.

The *MAD3* ORF was cloned into the Two Hybrid (pACTII, Table 2.1.9) vector containing the GAL4 DNA binding domain (Gal4DBD) by Kevin Hardwick producing plasmid pKH701. In this work the expression levels were assayed using the polyclonal anti-Mad3p antibody. A band was detected at approximately 70 KDa and was absent from the control lane (data not shown). This band relates to the chimeria of Mad3p and Gal4DBDp. A band corresponding to wild type Mad3p was also present in the extract.

The concentration of 3-AT required to reduce background was ascertained by transforming strain CG1945 (Table 2.1.7) with pKH701 and mating to the library strain. Diploids were selected for using the *TRP1* and *URA3* marker genes present in the CG1945 (containing the *GAL4-MAD3* plasmid) and Y187 (containing the FRYL library plasmid) strains respectively (See section 2.1.5). Equal measures of the mated culture were streaked onto selective agar plates containing different concentrations of 3-AT. The plates were incubated at 30°C for four days and the colonies were counted. A 3-AT concentration of 2.5mM was found to be the optimum concentration for the mating (data not shown).

5.4 Two-Hybrid Screen with pKH701

A full two-hybrid screen with pKH701 was carried out as described in Section 2.2.1.11. A total of 2.3×10^7 diploids were screened at a mating efficiency of 15%. Although the mating efficiency was relatively low, the number of diploids screened will provide inserts to cover the yeast genome at least once (See section 2.2.1.11.4), and the screen can be considered as saturating. The β-gal overlay assay produced a total of 109 colonies displaying a blue colour after 24 hours. Of these, 25 clones failed to grow

after re-streaking to fresh YMM -LWH medium, with a further 33 failing to reproduce the positive β-gal activity in a filter-lift assay.

The 51 clones that passed both test criteria *i.e.* His+ and LacZ+, were again restreaked onto fresh YMM-LWH medium. From these plates, the prey plasmids responsible for the two-hybrid interactions were rescued and plasmid DNA was prepared and sequenced (Section 2.2.3.7). The ORF encoded by each interacting plasmid, was determined using the BLAST search available at the SGD web site (see Section 2.2.4). The first nucleotide fused to the GAL4 DNA Binding Domain sequence was also determined. This allowed the identification of both the size and relative position of the fragment within the full length ORF. A total of 45 clones were successfully sequenced, however 6 clones either failed to give any colonies after plasmid rescue, or failed to produce sequence data. Table 5.1 summarises the information obtained from this screen.

5.5 Analysis of clones from pKH701 two-hybrid screen

The sequenced clones from the screen yielded several interesting putative Mad3p interacting proteins. The identification of Cdc20p in the screen is encouraging as the identification of a protein known to interact with Mad3p suggests the screen was successful and that the GAL4-Mad3p chimera was functional. The absence of Bub3p is surprising but could be due to Bub3p being non-functional itself when fused to a transcriptional activation domain or causing slow growth when over expressed.

Those clones deemed to be in the A1 category were analysed further for involvement in the mitotic checkpoint. In the first instance positives containing the same ORF were translated and aligned with the full length translated ORF (Figure 5.3). Deletion of the open reading frames found in the screen were tested for benomyl sensitivity (Figure 5.4).

Table 5.1

Gene	ORF	ORF size	Nucleotide from ATG	insert size	Category	Fig. 6.3 reference
None	YHR197c	non-coding		300	B	
None	YHR122w	695	244	486	A4	
MTH1	YDR277c	1301	338	533	A1	
SPB1		non-coding		483	B	
HTS1		non-coding		484	B	
None	YBR007c	2210	859	461	A4	
None	YKL153w	509	362	147	A4	
None	RDN	Intergenic		282	B	
None	YJR072c	non-coding		287	B	
None	RDN	Intergenic		406	B	
RTS1	YOR014w	2273	788	656	A1	RTS1B
ALR2	YFL050c	2576	626	614	A4	
SPO21	YOL091w	1829	579	557	A1	
RTS1	YOR014w	2273	866	575	A1	RTS1C
RTS1	YOR014w	2273	882	308	A1	RTS1E
None	YJR115	509	161	230	A1	YJR115wA
RTS1	YOR014w	2273	882	242	A1	RTS1F
None	YER033c	3230	1998	427	A4	
MTH1	YDR277c	1301	456	355	A1	
None	YJR115w	509	161	212	A1	YJR115wC
RTS1	YOR014w	2273	770	415	A1	RTS1D
MTH1	YDR277c	1301	445	477	A1	
SPO21	YOL091w	1829	482	566	A1	
MPH1	YIR002c	2981	1428	389	A1	
RIS1	YOR191w	4859	1142	560	A1	
SLN1	YIL147c	3662	3277	256	A1	
MTH1	YDR277c	1301	445	530	A1	
SLN1	YIL147c	3662	3198	259	A1	
SLN1	YIL147c	3662	3198	259	A1	
CDC20	YGL116w	1832	369	1360	A1	CDC20B
RIS1	YOR191w	4859	1159	1304	A1	
CAD1	YDR423c	1229	35	957	A1	
None	YOR066w	1889	2	830	A4	
MPH1	YIR002c	2981	1262	1871	A1	
MPH1	YIR002c	2981	1262	1871	A1	
SLN1	YIL147c	3662	2356	1176	A2	
None	YDR026c	1712	566	876	A2	
None	YDR026c	1712	517	927	A2	
CDC20	YGL116w	1832	374	1355	A1	CDC20A
CDC20	YGL116w	1832	374	1355	A1	CDC20A
None	YOR066w	1889	2	830	A4	
None	YJR115w	348	161	215	A1	YJR115wB
RTS1	YOR014c	2273	729	966	A1	RTS1A

Table 5.1 position and category of the clones found in two-hybrid screen using Mad3p

Figure 5.3a

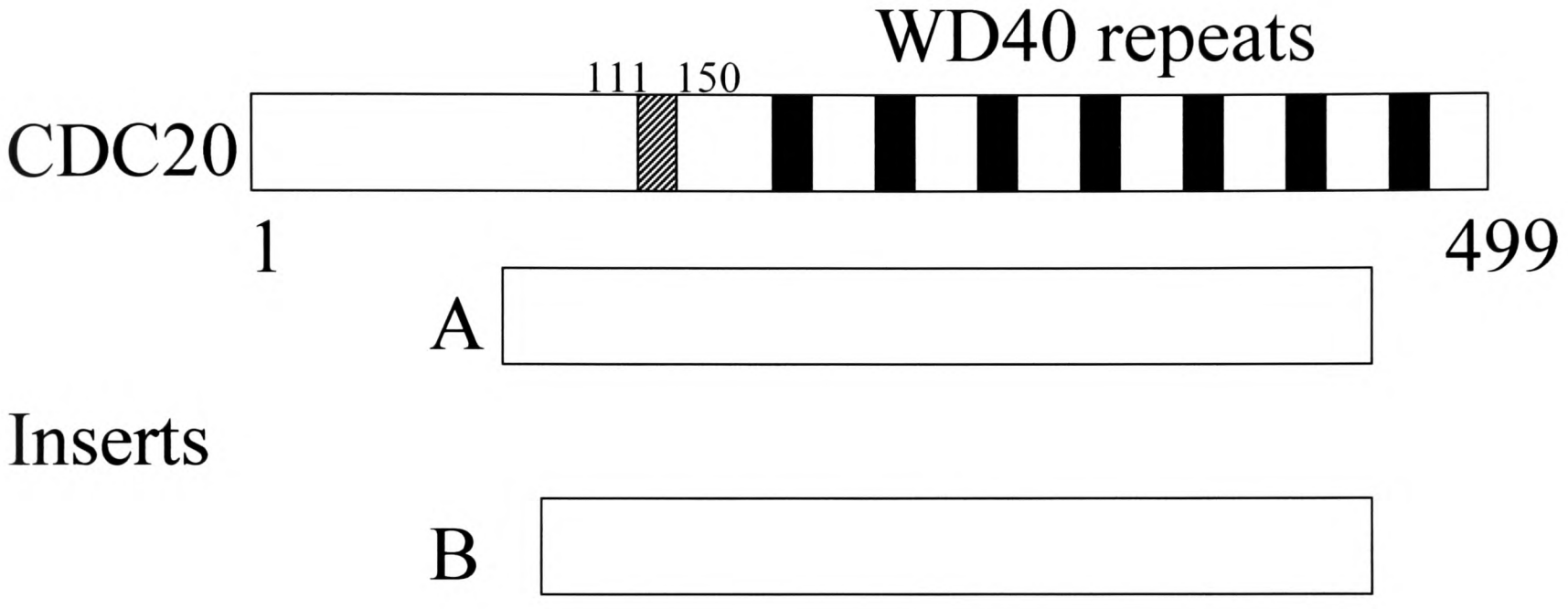


Figure 5.3 The CDC20 clones contain the Mad2p binding domain. The stripped box indicates the Mad2p binding domain of Cdc20p (Luo et al; Nature, March, 2000). As can be seen from the alignment this is present in theclones from the two hybrid screen.

Figure 5.3b

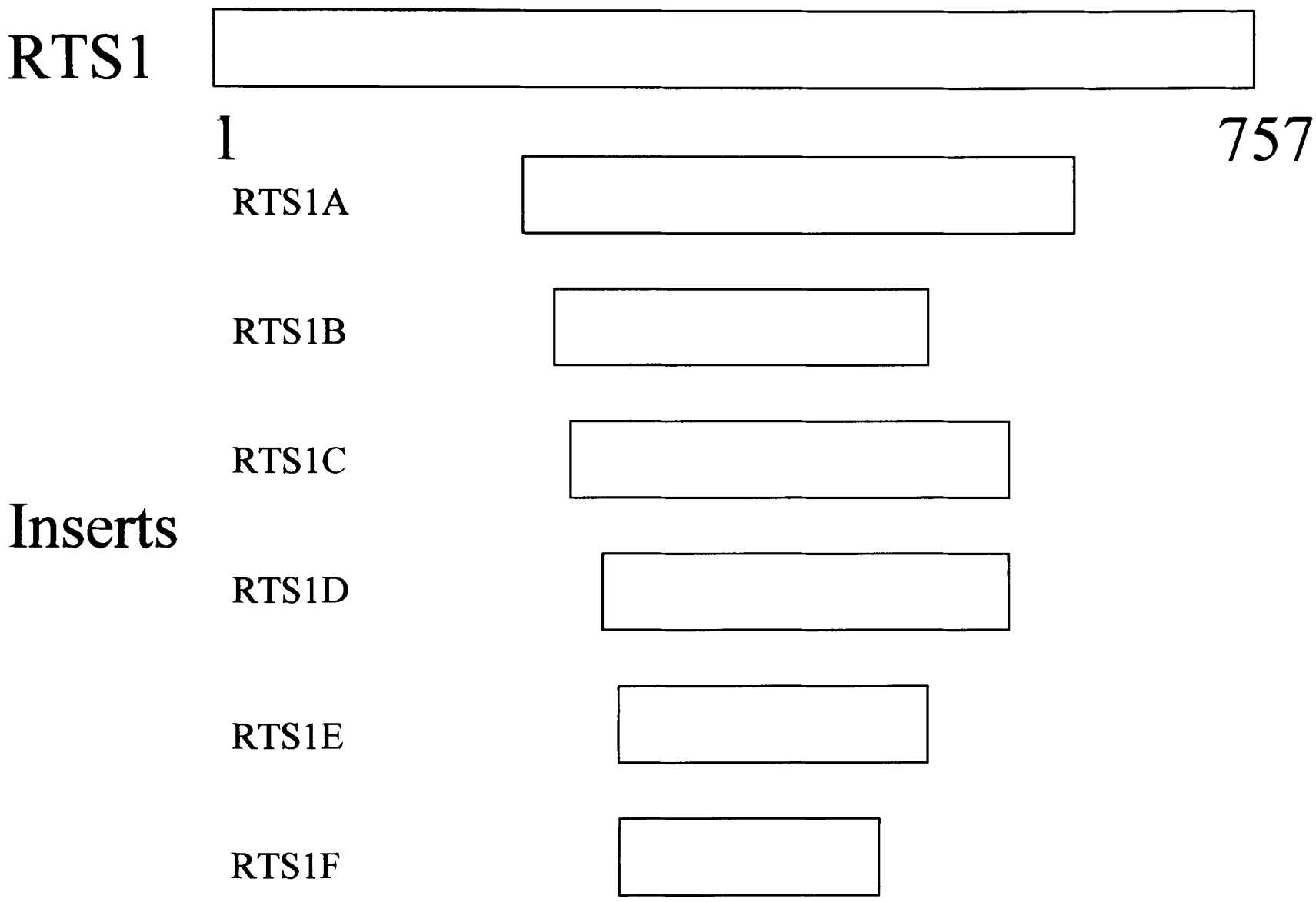


Figure 5.3b The RTS1 clones contain a common sequence. The RTS1 containing clones were translated and aligned against wild type RTS1. The RTS1F sequence is present in all six clones.

Figure 5.3c

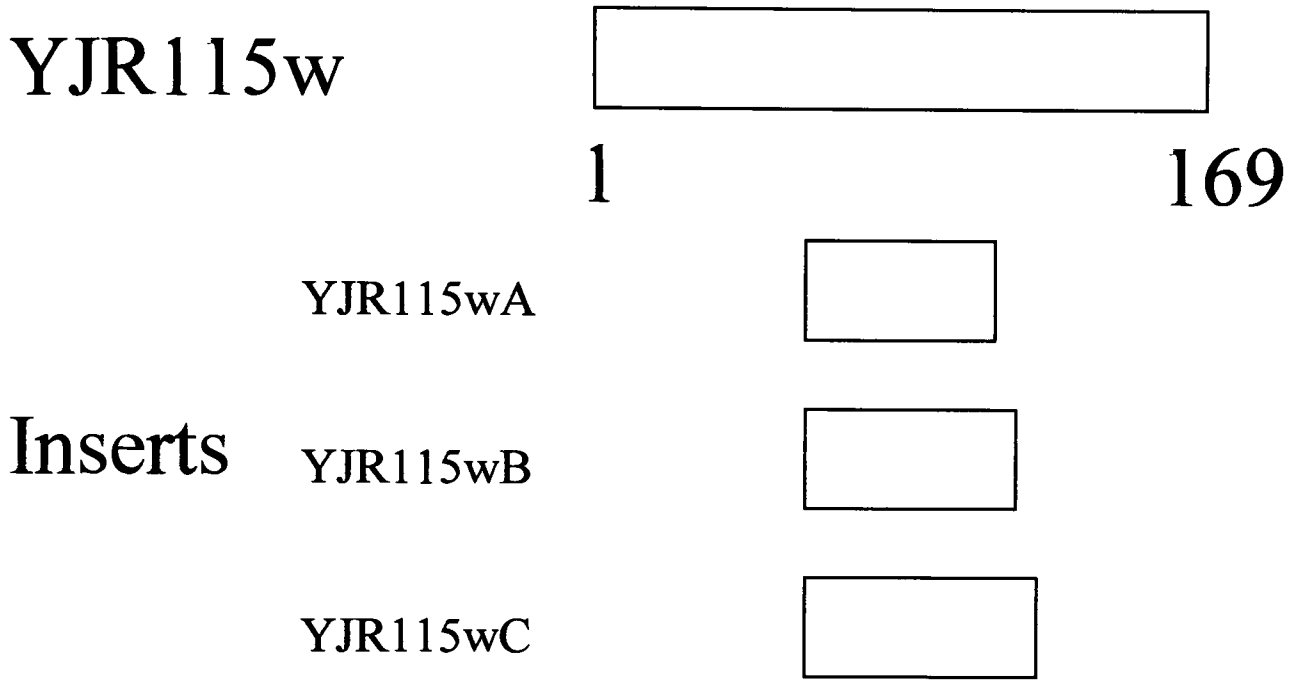


Figure 5.3c Alignment of YJR115w. The clones containing the YJR115w ORF were translated and aligned with the sequence for YJR115w obtained from SGD. No function for this potential protein have been found making it difficult to comment on the relevance of this finding.

Figure 5.4

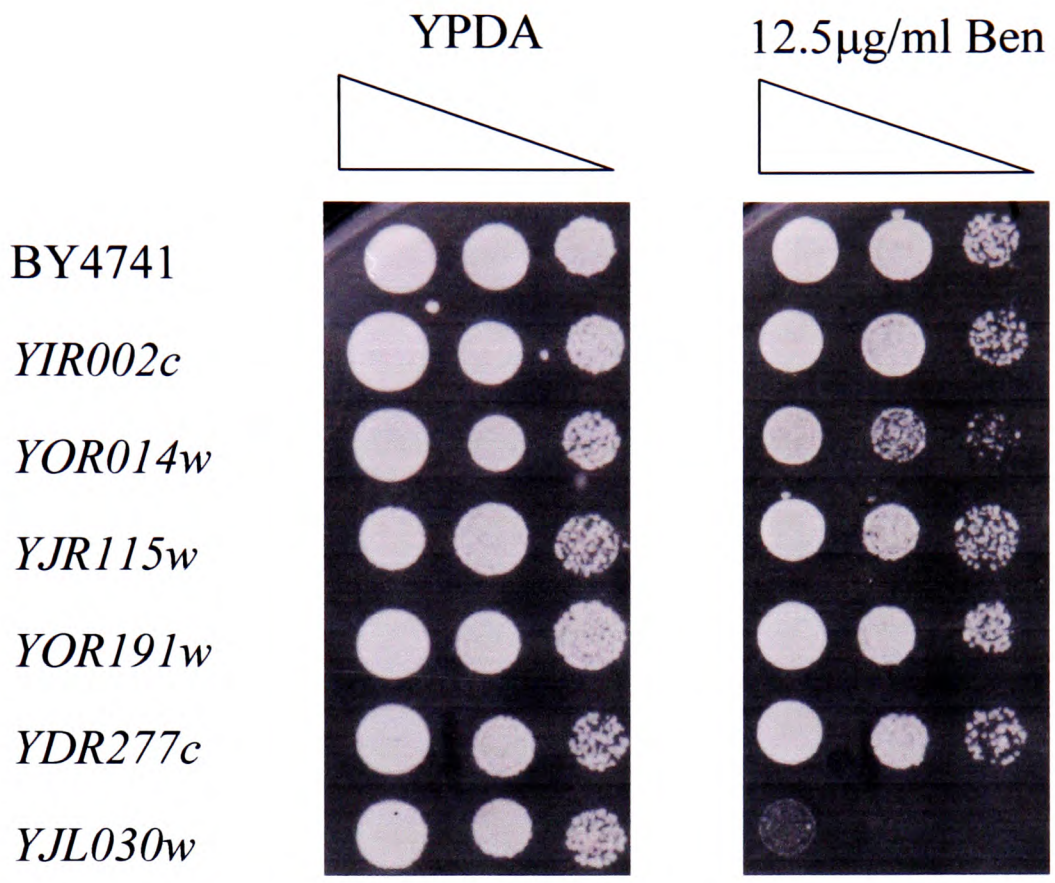


Figure 5.4 The benomyl sensitivity of a deletion of each of the ORF found in the screen. Deletions of the ORF found in the two-hybrid screen were obtained from EUROSCARF. A wild type strain, BY47541, and a *mad2Δ* strain, *YJL030w*, were used as controls. A dilution factor of 1 in 10 was used from left to right as symbolised by the triangles

5.5.1 Sequence alignment of fragments from clones obtained in screen.

The clones containing fragments of *CDC20* were translated into protein sequence and aligned with the full length protein (Figure 5.3a). The alignment shows the presence of the region of Cdc20p required for its interaction with Mad2p (Hwang, Lau et al. 1998) within all three clones. This is a significant result showing a common domain which is perhaps necessary for the interaction between Cdc20p and Mad2p, and Cdc20p and Mad3p. The identification of this domain in this screen suggests that Mad3p interacts with Cdc20p via Mad2p and this possibility is being tested using the two-hybrid approach.

Clones containing the fragments of *RTS1* were, again, translated into protein sequence and aligned with the full length protein (Figure 5.3b). The six fragments of *RTS1* found in the clones allow the identification of a potential Mad3p binding domain within the Rts1p. Fragment RTS1F shares a common sequence from residues 294 to 374 of Rts1p. Only clone RTS1A is in frame with the codons of the *RTS1* gene, however, this is not necessarily a problem for the two-hybrid system as it is possible for sufficient in-frame fusion protein to be translated due to ribosomal slippage to allow the identification of a positive interaction (Prof. Jean Beggs, Swann Building, King's Buildings, Edinburgh; personal communication).

The full length open reading frame of the potential ORF, YJR115w, was translated and aligned with translations of the clones found to contain fragments of this ORF. The potential significance of this discovery is difficult to measure due to the lack of knowledge concerning this open reading frame, and despite the *yjr115Δ* strain behaving in a wild type manner in the benomyl sensitivity experiment (Section 5.5.2), further analysis of the deletion strain may shed some light on its potential role in the mitotic checkpoint.

5.5.2 Benomyl sensitivity of deletions of a selection of the open reading frames.

Deletions of five of the ORF identified in the screen were ordered from EUROSCARF. Only these five were available from the database at the time of this work. A wild type, BY4741, and a *mad2Δ*, *YJL030w*, were also ordered to act as controls. The benomyl sensitivity was assayed as described in section 3.2.3 and repeated three times. Figure 5.4 shows the results of the analysis.

BY4741 was benomyl resistant and the other strains tested also displayed wild type growth with the exception of the *mad2Δ* strain, *YJL030w*, and the *rts1Δ* strain, *YOR014w*. The benomyl sensitive phenotype of the *rts1Δ* strain was subtle in comparison to the *mad2Δ* strain but was reproducible.

5.6 Discussion

The purpose of the two-hybrid screen was to identify novel interactions between Mad3p and other proteins, particularly those with relevance to the mitotic checkpoint. Bub3p and Cdc20p are two components of the checkpoint that were expected to be discovered in the screen. Indeed, several overlapping fragments of Cdc20p were found and will be discussed shortly. The absence of *BUB3* needs to be addressed as the biochemical interaction found *in vitro* and *in vivo* (Chapter 4, this work) make it the most obvious component to be found in a two-hybrid screen using Mad3p as bait.

Bub3p is not known to hinder cell growth when overexpressed (Chapter 6, this work) yet Cdc20p is (Hwang, Lau et al. 1998) and still colonies containing *CDC20* fragments being overexpressed from the prey plasmid were found in the screen. One explanation for the absence of *BUB3* is the very compact nature of its structure. Both Bub3p and Cdc20p are WD40 repeat proteins. These proteins generally contain a stretch of seven repeated sections of amino acids usually of 40 amino acids in length and each with a tryptophan and aspartic acid residue at their ends. The archetypal WD40 repeat protein is β-transducin where the seven repeats produced a propeller structure in the folded protein.

Bub3p was thought to contain only 5 of these repeating sections due to the shortness of the coding region and the identification of only 5 characteristic WD40 sections. However, recent structural work by (David Wilson, Section of Molecular and Cellular Biology, University of California, Davis; per. comm.) has confirmed the presence of all 7 repeating units and the characteristic three-dimensional propeller motif epitomised by β-transducin. This group also discovered that the entire length of Bub3p is sensitive to mutation so that even subtle changes in the primary sequence disrupts its structure and function. This sensitivity of Bub3p suggests that any fusion involving the protein would need to be of the full length protein. The probability of finding this fusion in a two-hybrid screen is lower than finding a fusion that could tolerate several missing amino acids.

Cdc20p is more tolerant. The first 250 amino acids are not involved in the propeller structure and it is in this region where a putative Mad2p binding site has been discovered (Hwang, Lau et al. 1998; Luo, Fang et al. 2000).

Figure 5.3a shows the presence of this domain within all of the fragments discovered in the screen. This suggests the possible requirement of Mad2p for the association between Mad3p and Cdc20p; a dependency already found in Chapter 4 of this work.

The second interesting discovery from this screen is that of *RTS1*. Rts1p is a regulatory subunit of the type 2A phosphatase of *Saccharomyces cerevisiae* and has recently been localised to the kinetochore (Shu, Yang et al. 1997; Gentry and Hallberg 2002). This interaction is immediately interesting as another PP2A regulatory subunit has been implicated in the mitotic checkpoint, Cdc55p (Minshull, Straight et al. 1996). As previously discussed in section 1.2.1 of this work, loss of *CDC55* abrogates the mitotic arrest of the checkpoint with concurrent inactivation of Cdc28p in the absence of cyclin degradation. It is possible that Mad3p interacts with Rts1p to somehow increase the "wait anaphase" signal during mitosis by stimulating or inhibiting the activity of PP2A. Although this is a tempting hypothesis, Zhao *et al* have shown that the roles of Cdc55p and Rts1p are functionally distinct and that over-expression of Rts1p in a *cdc55Δ* strain does not compensate for the mitotic defects seen in this strain (Zhao, Boguslawski et al. 1997). This involvement of Rts1p in the mitotic checkpoint is strengthened by its benomyl sensitive phenotype seen in figure 5.4 and its recent

localisation to the kinetochore (Gentry and Hallberg 2002). Certainly more work using the techniques described in chapter 3 of this work would assess this possible role for Rts1p.

Little can be said of the interaction between Mad3p and the ORF, *YJR115w*, as there is no information as to the function of its gene product. However, the interaction was found several times in the screen and was followed up by deletion of the open reading frame and analysis of its benomyl sensitivity. In the assay a strain containing a deletion of the *YJR115w* did not have a benomyl sensitive phenotype suggesting that the gene product of this ORF is not involved in the mitotic checkpoint although this could be due to redundancy between this gene and another. A genetic screen with this gene may provide more evidence for or against its role in the checkpoint or another role in mitosis.

The other positives found in the screen - *YIR002C* (*MPH1*), *YOR191w* (*RIS1*) and *YDR277c* (*MTH1*) - are of unknown importance as deletions were not found to be benomyl sensitive and all have diverse functions in protection from genome damage (Scheller, Schurer et al. 2000), chromatin assembly (Zhang and Buchman 1997), and glucose transport (Schulte, Wieczorke et al. 2000) respectively. If time allowed these genes, and *RTS1* and *YJR115w*, would have been tagged with an epitope such as that of MYC, and the subsequent chimeras would have been immunoprecipitated and the precipitates analysed for the presence of Mad3p and Mad2p. This work and other avenues of research must be left for other workers.

Since this work was finished a group of workers completed an exhaustive protein-protein interaction screen using both two-hybrid and TAP-tag methods. Mad3p was found to interact with Bub3p, Cln3p, Msb2p, Sap4p and Sin4p. Of these interacting proteins both Msb2p and Sap4p proteins also interacted with Mad2p (Uetz, Giot et al. 2000). The interaction with Sap4p is particularly interesting as this is also a member of the PP2A subunit family and has been shown to be involved in the transcriptional regulation of the yeast G1 cyclins; *CLN1*, *CLN2* and *CLN3* (Luke, Della Seta et al. 1996). This result should be followed with further investigation into the potential role of a mitotic regulator, Mad3p, in the entrance of yeast cells into the cell cycle.

In conclusion this chapter has opened other avenues for research concerning the potential roles for Mad3p in phosphatase regulation and G1 regulation via Rts1p,

and has identified a region of Cdc20p that may be required for the interaction between the two proteins. Other workers will no doubt pursue this line of enquiry and by using available point mutants of *CDC20* and/or additional two-hybrid screens using specific portions of Cdc20p.

Chapter 6 - Distinct Chromosome Segregation Roles for mitotic checkpoint proteins

6.1 Introduction

The loss or gain of all or part of a chromosome could lead to or propagate a cancerous cell. The mitotic checkpoint prevents the unequal segregation of sister chromatids and so protects against the loss or gain of chromosomes. The checkpoint genes are non-essential in yeast but not in multicellular organisms. The loss of Mad2 from the worm, *Caenorhabolitis elegans*, and mice leads to early embryonic lethality and the loss of Bub1 from *Drosophilia* also causes death at the early embryonic stage of development (Mad2, (Dobles, Liberal et al. 2000); (Kitagawa and Rose 1999); Bub1, (Basu, Bousbaa et al. 1999)). The embryos display massive chromosome loss in these mutants. This points to the importance of the mitotic checkpoint in maintaining correct ploidy (the correct number of chromosomes) in dividing cells. The identification of spindle checkpoint mutants in cancers is not surprising given this data and Cahill *et al* found mutant alleles of Bub1 in a subtype of human colorectal cancer cells (Cahill, Lengauer et al. 1998). Two phenotypes of lung cancer cells were analysed in the work; MIN (microsatellite instability) and CIN (chromosome instability). The MIN phenotype is caused by an increased mutation rate at the nucleotide level and the CIN phenotype is characterized by the loss of correct ploidy (aneuploidy). The CIN phenotype was found to harbor mutations within the Bub1 gene. By transforming non-CIN cells with the mutant Bub1 allele Cahill *et al* were able to show that this mutation alone can dominantly cause a CIN phenotype. The dominant effect of the mutant allele is critical in cancer onset as it means only one copy of the BUB1 gene in human cells can cause aneuploidy.

Since Cahill *et al* reported their findings there have been studies identifying mutations in hMAD1 and hMAD2 in cancer cell lines and tumours (Percy, Myrie et al. 2000) and the human homologue of Mad3p, BubR1/Mad3L, was found to be relevant in BRCA2 mutant breast cancers (Li and Benezra 1996).

This chapter aims to find the degree of aneuploidy of the spindle checkpoint mutants using the powerful genetics of *S. cerevisiae* and a method for assaying the loss of a marked chromosome from a population of yeast.

6.2 The Chromosome loss assay

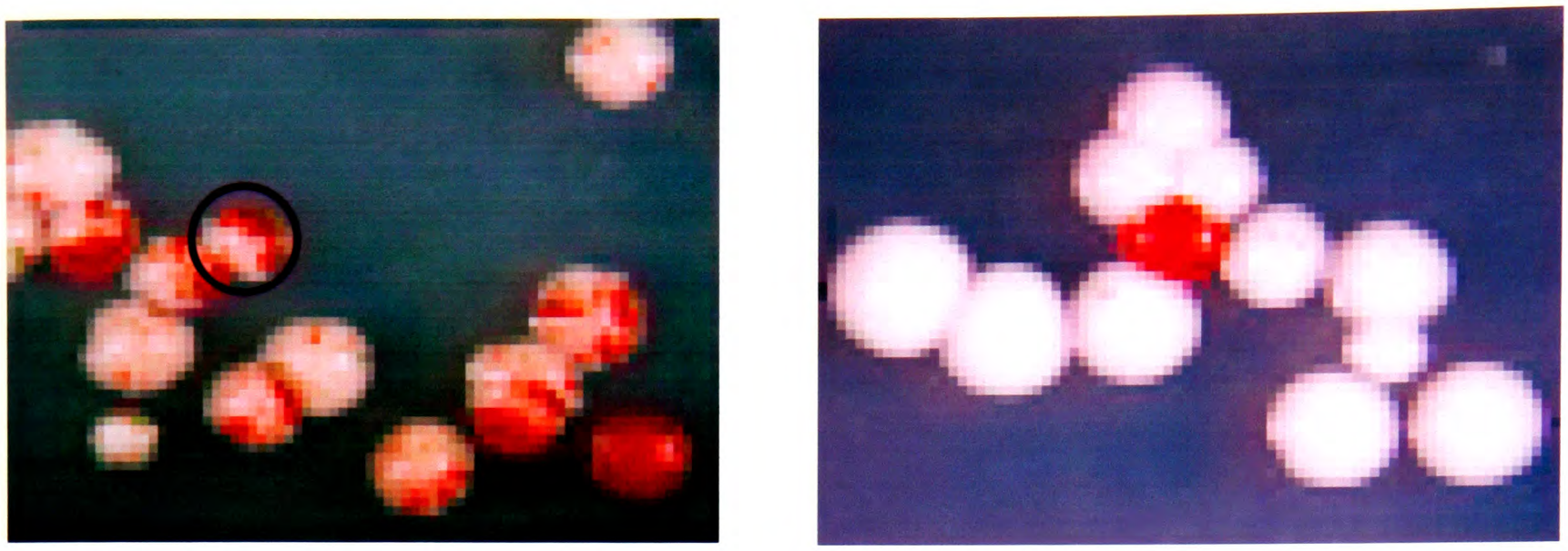
Hieter *et al* devised a system to assay the rate of chromosome loss in a yeast strain containing an ochre mutation in the *ade2* locus (Hieter, Mann et al. 1985). The *ade2* mutation makes cells auxotrophic for adenine which is supplemented in the growth media allowing cells to grow, however, the mutation produces a red coloured yeast colony due to the accumulation of a biosynthetic intermediate in the yeast vacuole. The STOP codon can be suppressed by a tRNA which recognises the STOP codon as encoding for an amino acid allowing the Ade2p to be made. The presence of this suppressor gene, *SUP11*, in the yeast strain produces a white coloured colony because the red intermediate no longer accumulates.

Hieter *et al* utilised this system to assay chromosome loss by integrating the *URA3* and *SUP11* genes into a copy of chromosome III (Hieter, Mann et al. 1985). This artificial chromosome is not required for viability on rich growth medium. If a colony contains the chromosome it will be white, colonies lacking the chromosome are red. In order to assay the number of chromosome mis-segregation events in a population of yeast the strain containing the *URA3 SUP11* chromosome and the genetic element whose effects on chromosome segregation are being investigated eg *mad3Δ*, is grown in liquid culture to log phase. A sample of the liquid culture is diluted and plated onto YPD plates. These plates contain high enough adenine to ensure sufficient growth of the strain and low enough to allow the colonies to become coloured. After several days the colonies are counted and their colour is noted.

As one colony arises from one cell, if the colony is white then it contained the *URA3 SUP11* chromosome when it first began to divide. If the colony is red then it must have lost it before it began to divide on the plate. However, if the colony is half red and half white then the *URA3 SUP11* chromosome was lost in the first division that the cell made when it began to divide on the plate (Figure 6.1). By counting the number of half red/half white colonies and dividing this by the total number of colonies on the plate and multiplying by 1000, the chromosome loss rate per 1000 cell divisions can be arrived at.

The assay was performed by growing the cultures to be assayed overnight to attain a log phase culture the following day. Once log phase was attained at an OD_{600}

Figure 6.1



bub1Δ *wt*

Figure 6.1 - A visual assay to monitor chromosome loss. The left panel shows the chromosome loss in a *bub1Δ* strain (YRJ 112), with the right panel an example of a wt (KH231) strain in the same assay. Compare the half sectored colonies circled in the *bub1Δ* panel with the unsectored colony circled in the wt panel.

of between 0.3 -0.5 the cultures where left to grow for 2 hours. The cultures were then diluted and plated on YPD plates to give approximately 500 colonies per plate to facilitate counting.

This assay was used to score the chromosome loss rates of checkpoint mutants, over-expressed checkpoint components and heterozygous diploid checkpoint components in this study.

6.2.1 Spindle checkpoint mutants have a range of chromosome loss rates

A wild type yeast strain containing the marked chromosome (KSO41) was crossed to deletion mutants of *MAD3* and *BUB1,2,3* and the diploids were sporulated and spores containing the marked chromosome and the spindle checkpoint mutant were selected. D.M.Brady performed the cross using *mad1Δ* and *mad2Δ* strains.

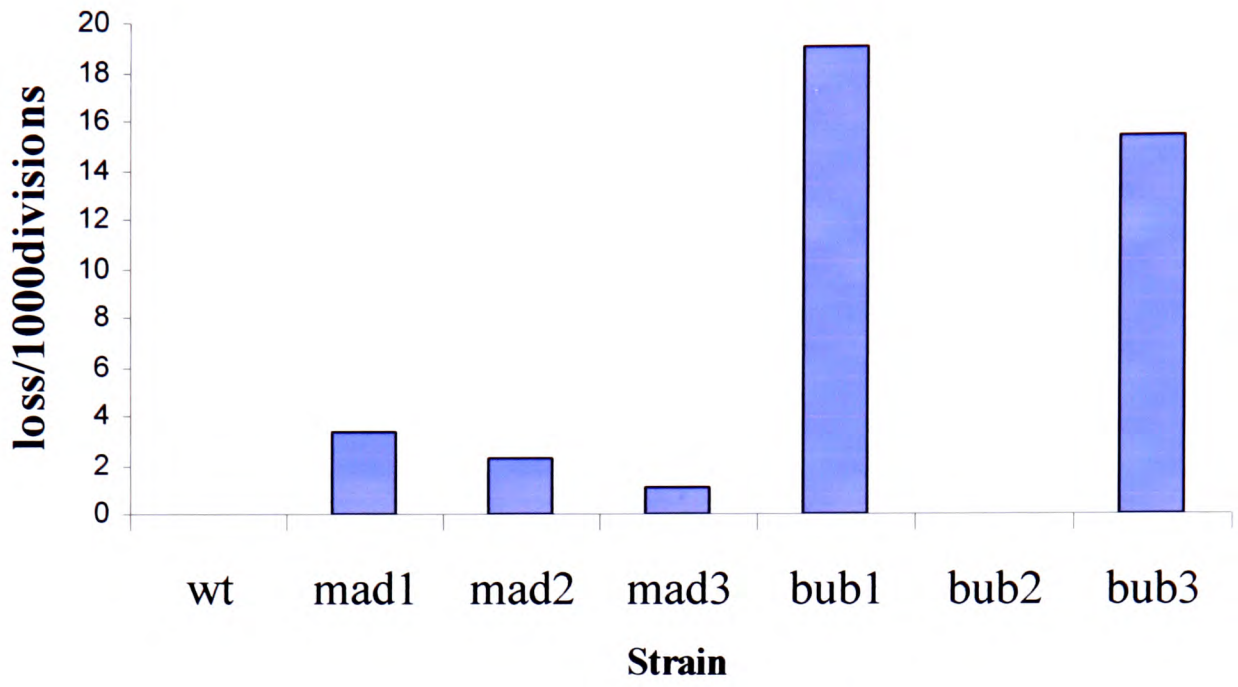
The chromosome loss assay was performed as described and the loss rates were calculated for the 10000 colonies counted for each mutant. Figure 6.2a shows the chromosome loss rate per thousand cell divisions of the spindle checkpoint mutants. The chromosome loss rates of the spindle checkpoint mutants differs from mutant to mutant, it is possible to categorise the spindle checkpoint components by their chromosome loss rates into three categories: 1) *BUB1* and *BUB3* are most important for chromosome transmission. 2) *MAD1*, *MAD2* and *MAD3* form a second group with a significant chromosome loss rate relative to wild type but markedly less than that of *bub1Δ* or *bub3Δ*. 3) The chromosome loss rate of the *bub2Δ* strain is equivalent to that of a wild type strain. The level of benomyl sensitivity of the checkpoint components can also be categorised in a similar manner (Figure 6.2b). The only exception is *bub2Δ* which has the same level of benomyl sensitivity as the *mad* mutants.

6.2.2 Chromosome loss rates of *mad3* mutants

The chromosome loss rate of the *mad3Δ* strain was lower than that of the other mad mutants. The loss rates of the region I and region II mutants discussed in Chapter 4 may provide a different result. As *bub1Δ* and *bub3Δ* produced the highest level of

Figure 6.2

A



B

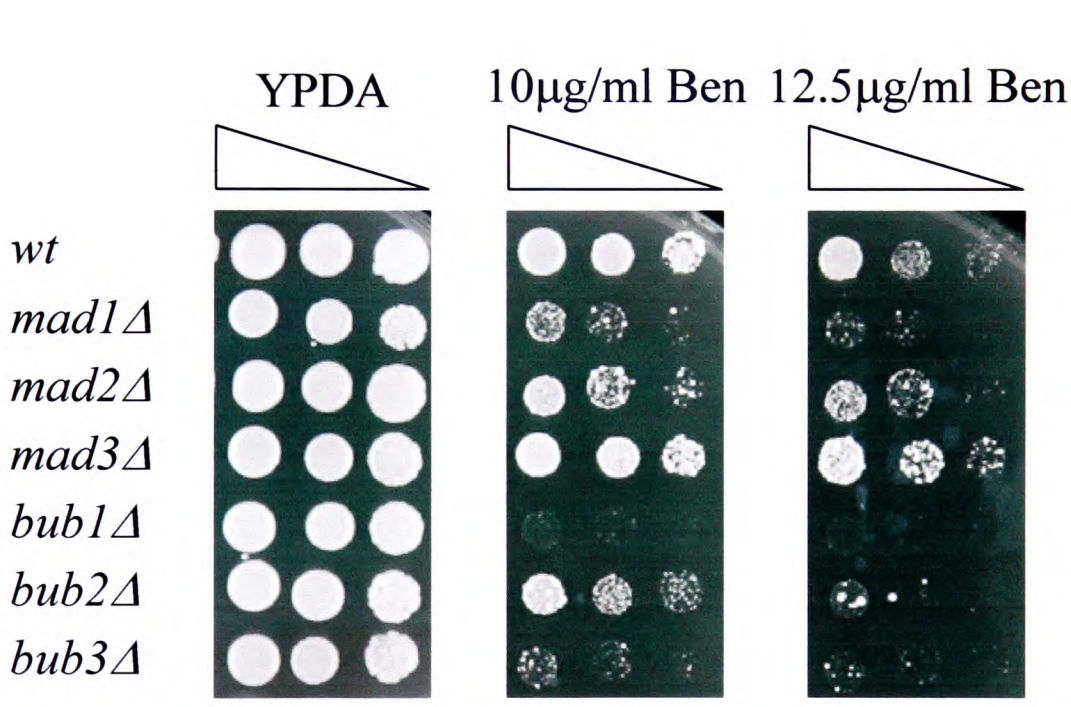


Figure 6.2 - Differing chromosome loss rates and benomyl sensitivity of spindle checkpoint mutants. (A) *bub1Δ* and *bub3Δ* are 16-18 fold more benomyl sensitive than wt cells. *mad1Δ* and *mad2Δ* strains are 2-3 fold more sensitive than wt cells and *mad3Δ* shows a low sensitivity. *bub2Δ* strains are equivalent to *wt* cells. These data are the combined results of 3 experiments. (B) A similar pattern emerges for benomyl sensitivity with *bub1Δ* and *bub3Δ* being the most sensitive but in this case *mad3Δ* is the least sensitive, not *bub2Δ*. Ten fold serial dilution were plated onto each plate.

chromosome loss could the shared domains between Bub1p and Mad3p be implicated in the loss rates? To test this hypothesis the region II mutant was rescued into a *TRP1* marked plasmid by gap repair. This method involved cutting a *TRP1* plasmid containing the *MAD3* gene (pKH535), driven from its own promoter, with restriction enzymes on either side of the mutation. This linearised plasmid was transformed in *mad3-1* and Trp^+ colonies were selected and the plasmid rescued. The plasmid was sequenced to confirm the presence of the region II mutation, this created plasmid pRJ22 (Section 2.2.3.7). The plasmids were transformed into strain YRJ111. A *TRP1* marked plasmid containing the *MAD3* gene was transformed into the strains as a control for expression from the plasmid. The plasmid vector missing the *MAD3* ORF was also transformed into strain YRJ111 as a negative control. Figure 6.3a confirms the benomyl sensitivity of the plasmids which has already been shown for pRJ21 (Figure 4.4b). The chromosome loss rates of the mutants was not significant compared to *mad3Δ* (Figure 6.3b) after the chromosome loss frequency was calculated from 5000 colonies counted for each mutant.

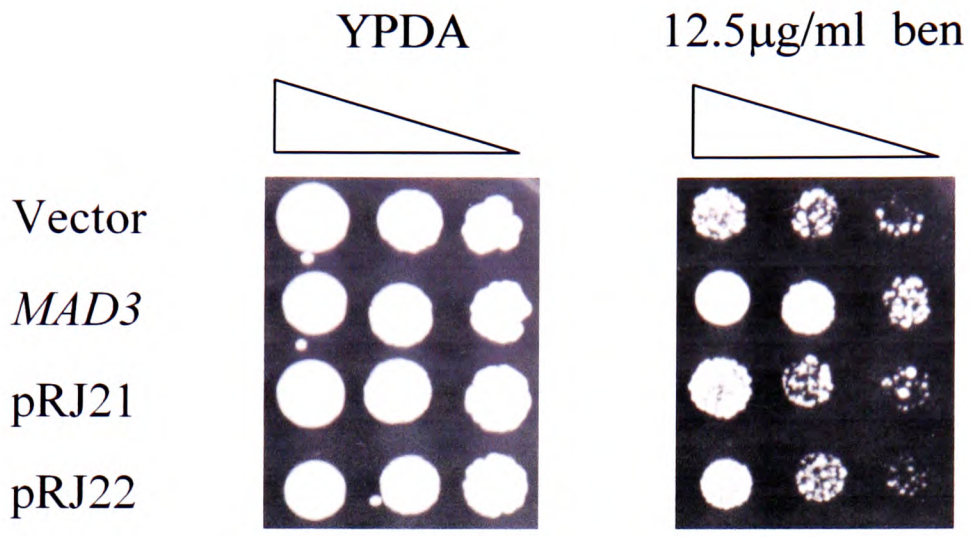
6.2.3 Over-expression of *MAD3* and the NH_2-terminus of *BUB1* and causes chromosome loss.

MAD1,2,3 were cloned into a 2μ plasmid containing the methionine repressible promoter creating plasmids, pKH301, pKH302 and pKH303. *BUB3*, *BUB1* and fragments of *BUB1* were cloned into the same plasmid by C.Warren (John Hopkins School of Medicine, Baltimore, MD 21205). These plasmids were each transformed into KSO41 and the cultures were grown overnight in selective media. The following day each culture was diluted back to an OD_{600} of 0.3 - 0.5 and allowed to recover for 2 hours. The cultures were then transferred to media lacking the amino acid, methionine, and grown for a further 2 hours. The assay was then carried out as described and the loss rates calculated from 3000 colonies counted for each plasmid.

Both Mad3p and the NH_2-terminus of Bub1p cause chromosome loss when over-expressed at levels above those of empty vector (Figure 6.4, compare data for MAD3 and N608 to pCD179). The low level of BUB1 chromosome loss rate compared to fragments of Bub1p may be due to lower stability of the full length protein.

Figure 6.3

A



B

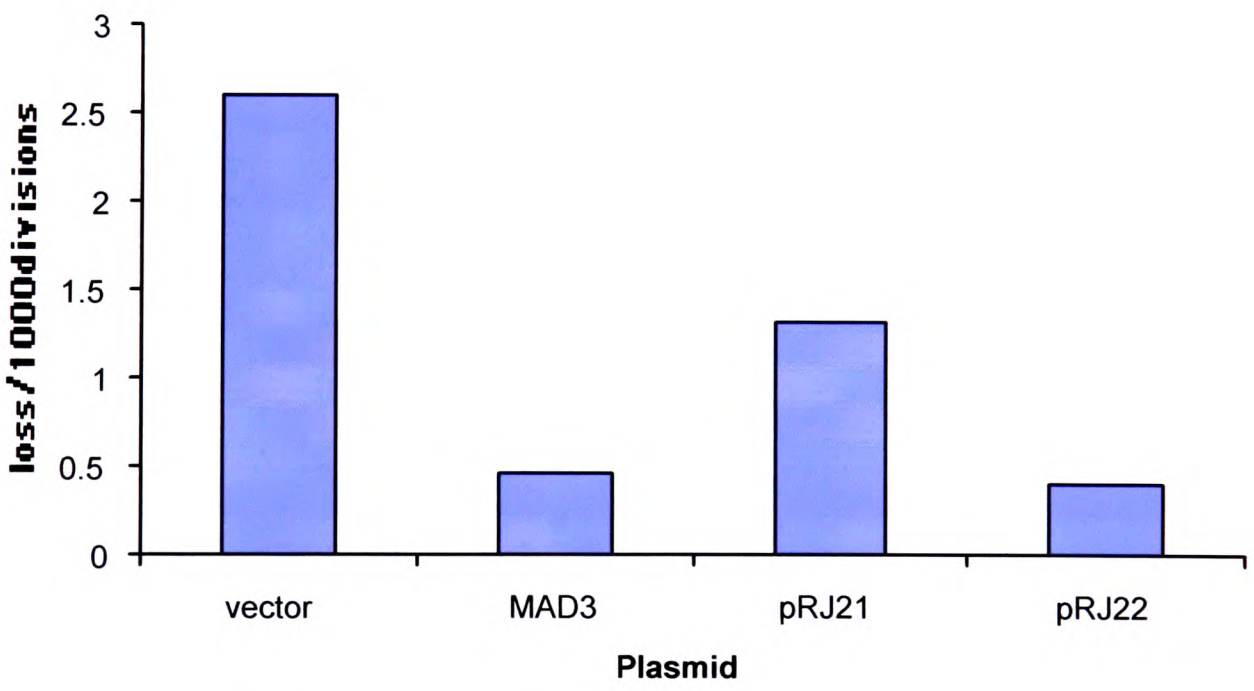


Figure 6.3 - Plasmid borne mutations within region I or region II of *MAD3* are benomyl sensitive but have a subtle chromosome loss phenotype. A) a plasmid containing *MAD3* rescues the benomyl sensitivity of a *mad3Δ* (compare *MAD3* to vector). Neither pRJ21 (region I mutant) nor pRJ22 (region II mutant) rescue the benomyl sensitivity of a *mad3Δ*. B) pRJ21 shows a 2-fold increase in chromosome loss over pRJ22, however, this is yet lower than the loss rate of a *mad3Δ* (vector).

Figure 6.4

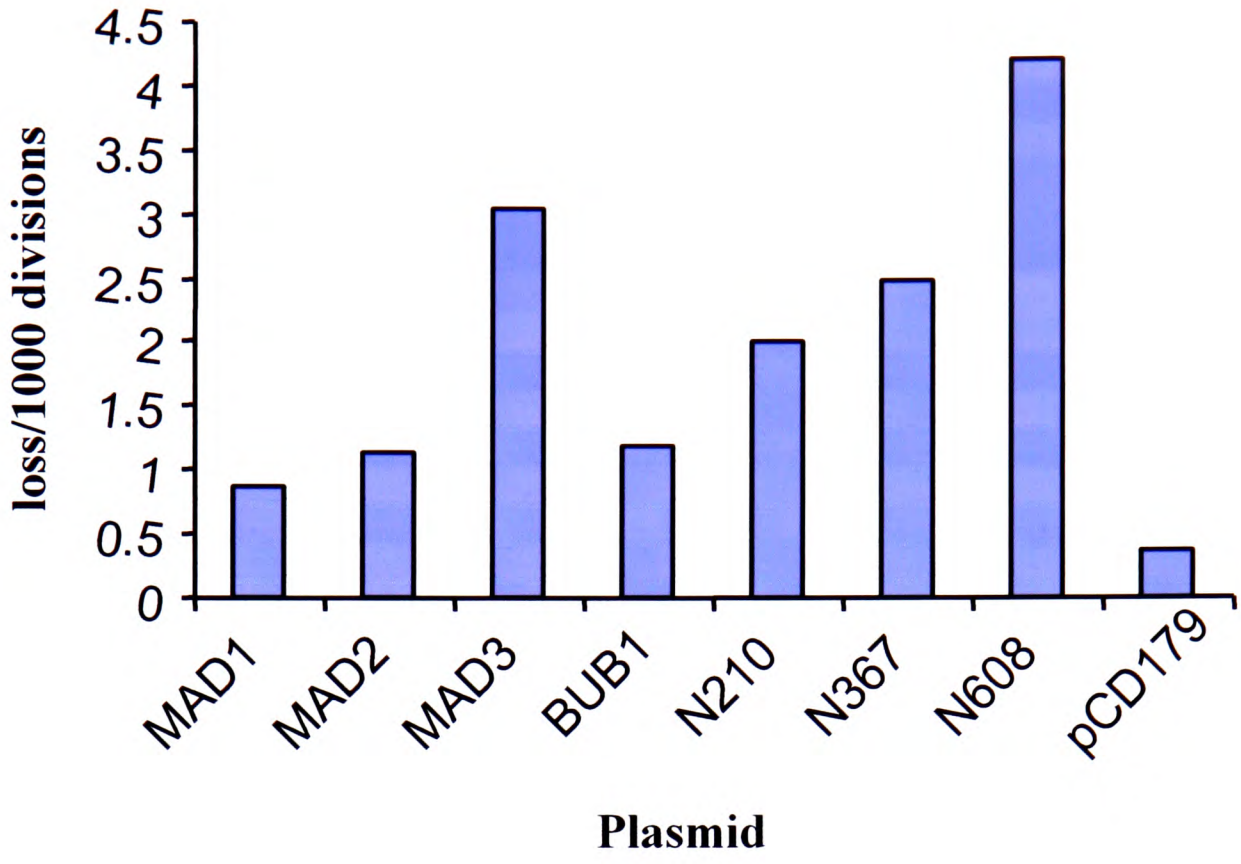


Figure 6.4 - All lanes correspond to the gene being over-expressed. The plasmids used are from left to right (section 2.1.10): pCD46, pCD47, pCD48, pCD44, pCD49, pCD50 and pCD51. pCD179 is an empty vector control. *MAD3* and *N608* over-expression cause chromosome loss in the KSO41 strain. N210, N367 and N608 represent plasmids containing the first 210, 367 and 608 amino acids. V179 The checkpoint gene was expressed by growing in -Methionine medium for 2hrs at 30°C. *MAD3* and the NH_2-terminal half of *BUB1* showed the most significant chromosome loss.

6.2.4 Haplo-insufficiency is relevant to diploid yeast

A recent paper found an increase in chromosome instability in mouse cells containing only one copy of the mMAD2 gene (Michel, Liberal et al. 2001). This haplo-insufficiency phenotype would be relevant to carcinogenesis as the loss of one copy of a checkpoint component is a single event much like the transformation of one copy of a checkpoint gene into a dominant negative allele. Aneuploidy would only take one mutation in each case. Could the loss of one copy of a checkpoint gene in a diploid *S. cerevisiae* strain cause chromosome loss?

Diploids heterozygous for the spindle checkpoint components, *MAD1,2,3*, were created by mating deletion mutants of the components with KSO41. A homozygous *MAD* strain was created by mating a and α KSO41 strains. A homozygous *mad* strain was created by mating a and α YRJ111 strains.

The chromosome loss assay was performed as described and the loss rates were calculated from 5000 colonies counted for each diploid. Figure 6.5 shows the loss rates for the different strains. The *mad2*/MAD heterozygote lost 3-fold more chromosomes than the MAD/MAD diploid. This is a significant increase and suggests that haplo-insufficiency is relevant to the stability of yeast chromosomes as well as murine chromosomes. Figure 6.5 also shows that *mad3*/MAD heterozygous cells lose chromosomes at a higher rate than MAD/MAD dipoids but both *mad2* and *mad3* heterozygotes are less susceptible to losing chromosomes than the *mad3/mad3* homozygous mutant. This suggests a dosage dependence upon the chromosome stability of mitotic checkpoint components. Again the relative importance of the checkpoint components in chromosome stability is called into question as *mad1*/MAD cells have a similar loss rate to that of MAD/MAD diploids.

6.3 Discussion

The results in this chapter suggest different roles for the mitotic checkpoint components in the maintenance of chromosome stability.

The data from Figure 6.2 show a *wt* level of chromosome loss for the *bub2Δ*. This is unsurprising as Bub2p has recently been shown to be involved in mitotic exit and not directly involved in the mitotic checkpoint (Daum, Gomez-Ospina et al. 2000;

Figure 6.5

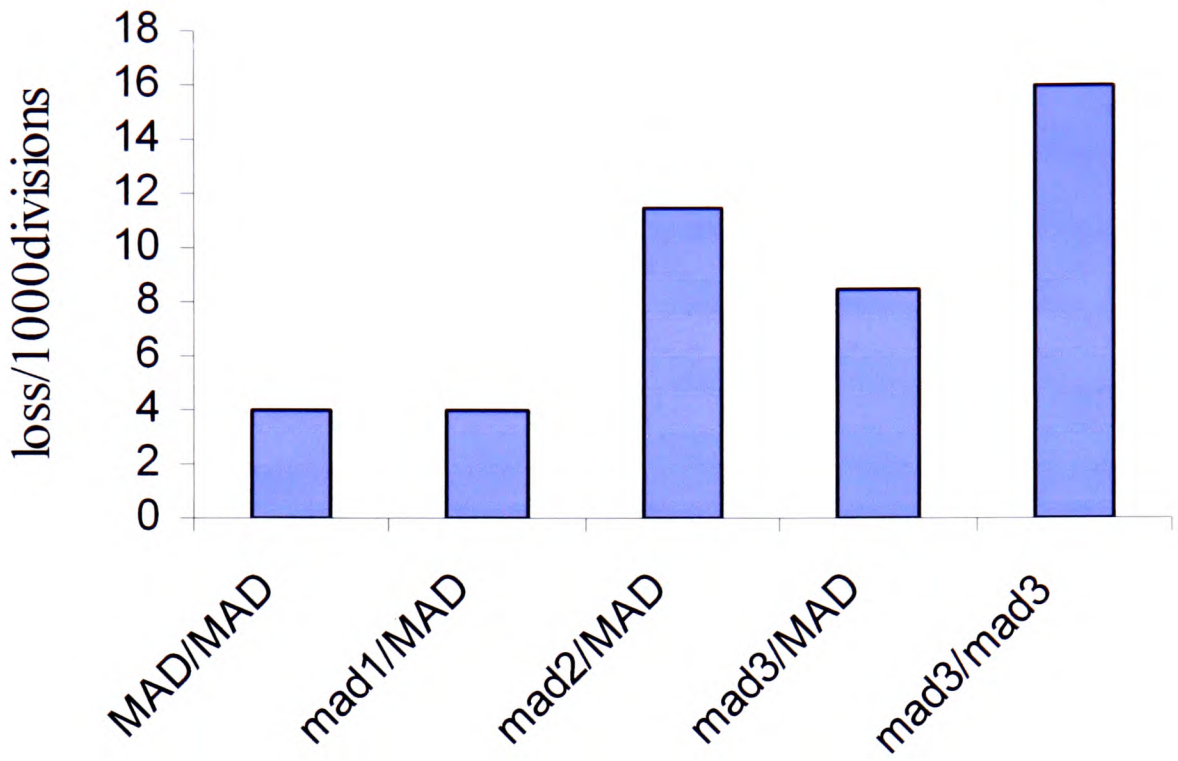


Figure 6.5 - Heterozygous checkpoint mutants lose chromosomes at a higher rate than wild type diploids. The *mad2* and *mad3* heterozygous diploids (*mad2*/MAD and *mad3*/MAD respectively) lose chromosomes between 2-3 fold more than the wild type diploid (MAD/MAD). The homozygous checkpoint mutant (*mad3*/*mad3*) loses chromosomes at a higher rate than heterozygotes, compare *mad3*/*mad3* to *mad3*/MAD3.

Krishnan, Pangilinan et al. 2000; Wang, Hu et al. 2000). In budding yeast both anaphase and mitotic exit require the activity of microtubules explaining why *bub2Δ* strains are benomyl and nocodazole sensitive (Hoyt, Totis et al. 1991).

The separation of the other checkpoint components into the groups indicated (section 6.2) is surprising as it indicates that the mitotic checkpoint components do not fit into a single pathway which detects and responds to a single signal as has previously been thought as illustrated in figure 1.6 (Hardwick, Weiss et al. 1996). The different groups correlate with constitutive complexes found within the checkpoint components. The Bub1p/Bub3p and Mad1p/Mad2p complexes are present throughout the cell cycle (Chapter 4; Figure 4.6 and (Chen, Brady et al. 1999) respectively). The low level of chromosome loss in the *mad3Δ* suggests a different role for the Mad3p/Bub3p complex. It must be concluded that the Bub1p/Bub3p association is vital for the fidelity of chromosome stability.

The over-expression of the checkpoint components also results in chromosome instability although to a lesser degree than the absence of the components (Figure 6.4). This low level of instability makes it difficult to comment on these results but the same experiment performed by our collaborators yielded better results. This could be due to the different media used or a slight variation on the protocol described here (Section 6.2). Warren *et al* obtained a loss rate of up to 20-fold above control for the NH_2-terminal 608 amino acids of Bub1p (N608). The group expressed only region II of Bub1p (Figure 4.1) in the chromosome loss assay and obtained a similar result as for the N608 fragment. As shown by others this region of Bub1p is responsible for the Bub1p/Bub3p complex formation (Roberts, Farr et al. 1994). By over-expressing *BUB3* in cells containing the over-expressed *BUB1* N608 fragment the group were able to rescue the chromosome instability phenotype. The interaction between Bub1p and Bub3p is clearly important for the maintenance of chromosome stability. Mad3, Mad2, Mad1, Bub3 and Bub1 and have all been localised to the kinetochore of higher eukaryotes (Jin, Spencer et al. 1998; Taylor, Ha et al. 1998; Martinez-Exposito, Kaplan et al. 1999; Chen 2002; Ikui, Furuya et al. 2002). Perhaps the absence of these components from the kinetochore affects kinetochore function to different degrees or could there be different signals transmitted by the different components. One possibility is the detection of tension and attachment at the kinetochore. Mad2p has

been shown to respond to the absence of attachment to the mitotic spindle and Bub1/BubR1 has been shown to respond to an absence of tension in mammalian cells (Skoufias, Andreassen et al. 2001).

Not only can the over-expression of a mitotic checkpoint component cause chromosome loss but the lowered expression of a component can also lead to chromosome instability. Figure 6.5 shows this and places more importance upon the levels of Mad3p within the cell. A confusing result for Mad3p when compared to the loss rates of the single deletions in section 6.3. The result for the *mad2*/MAD confirms work done in murine cells showing chromosome instability in mice heterozygous for *MAD2*. This is also relevant to tumourigenesis as heterozygosity of genes is a common occurrence in cancer cells (Dos Santos and Van Kessel 1999).

Taken together the results in this chapter outline a role for the mitotic components in the maintenance of chromosome stability. These results are analogous to the increase in loss of chromosomes and chromosome fragments in cancer cell lines. The results obtained here for Bub1p and Bub3p indicates weak points within the mitotic checkpoint that may be targeted by cancer cells in early carcinogenesis. The early identification of mutations in the human homologues of these genes may identify people with an increased rate of chromosome instability and as such an increased chance of developing cancer.

Chapter 7 - Final Discussion

7.1 Mad3p has a checkpoint function

The mitotic checkpoint of *S. cerevisiae* and indeed all eukaryotes plays a vital role in the transmission of genetic material from one generation to the next. The components of this checkpoint were initially identified as being the products of 6 genes; *MAD1,2,3, BUB1,3* and *MPS1*. Mutations in these genes result in the sensitivity to microtubule depolymerising agents, the premature segregation of sister chromatids in response to spindle damage, and an increased rate of division in the presence of microtubule depolymerising agents (Rudner and Murray 1996). This work confirms the benomyl sensitivity of *mad3* mutants and the higher rate of cell division in these mutants. The phenotypes evident in the *mad3* mutants were mapped to the YJL013c locus and sequencing of this locus identified mutations in the coding sequence. A deletion of the YJL013c reading frame made cells benomyl sensitive in a manner similar to that of a *mad1Δ* but different to the phenotype of a structural/functional component of the mitotic spindle (Hardwick and Murray 1995). This distinction is important as a mutation in a structural/functional component of the mitotic spindle may also make cells sensitive to an additional stress on the spindle such as spindle disruption. Therefore, benomyl sensitivity isolates mutants involved in all parts of spindle assembly and function. The microcolony assay, described in figure 1.8, can narrow this set of mutants to those that continue to divide in the presence of benomyl. Further evidence from other organisms supports a role for Mad3p in the mitotic checkpoint. The *MAD3* gene from *S. pombe* and humans is also involved in the mitotic checkpoint (Taylor, Ha et al. 1998; Millband and Hardwick 2002). The evidence presented in this thesis and the supporting evidence from others confirms that the YJL013c locus encodes the mitotic checkpoint component, Mad3p.

7.2 Mad3p is a nuclear protein

Mad3p was localised to the nucleus of cells over-expressing the *MAD3* gene. Unfortunately the subcellular location of Mad3p could not be narrowed down further in this study although evidence from mammalian cells concerning the homologue of Mad3p, BubR1, found this protein to be present on the kinetochore of mitotic cells with an increased staining on unattached kinetochores (Taylor, Ha et al. 1998) (Taylor, Hussein et al. 2001). Not only has BubR1 been localised to the kinetochore itself but it is also required for the correct localisation of Bub1, Bub3, Mad1 and Mad2, and the kinesin protein, CENP-E (Chen 2002). Mad3 has also been localised to the kinetochore of fission yeast (Millband and Hardwick 2002).

Millband *et al* over-expressed Mad2 in fission yeast, and by doing so induced a metaphase arrest. This arrest was only dependent on the presence of Mad3 within the cell so it is possible that Mad3p localises to the and functions at the end stage of checkpoint activation. Future work using more powerful localising techniques will undoubtedly find Mad3p at the kinetochore of *S. cerevisiae*.

7.3 Mad3p has an effector role in the mitotic checkpoint

Evidence from epistatic experiments using a *GAL-MPS1* strain places Mad3p at the effector end of the mitotic checkpoint pathway (Hardwick, Weiss et al. 1996). Mad1p is phosphorylated in checkpoint active cells and this phosphorylation also occurs in cells over-expressing the *MPS1* protein kinase (discussed in section 1.2.1 and illustrated in figure 1.6) (Hardwick, Weiss et al. 1996). Over-expressing *MPS1* in mitotic checkpoint mutants identified components necessary for this Mad1p phosphorylation and components dispensible for this modification. Mad3p was found to be dispensible for the phosphorylation of Mad1p suggesting it functions after Mad1p in the checkpoint. A linear pathway was constructed using this data (Figure 1.6). The simplicity of this model is tempting but work since its inception, including

chapter 4 of this thesis, has eroded the idea of a single signal transduction pathway and instead points towards a more complex strategy for mitotic checkpoint function. The interaction between Mad3p and Bub3p shows an interaction between molecules found both before and after Mad1p phosphorylation.

In Chapter 4 an interaction between Mad3p and the target of the mitotic checkpoint, Cdc20p, was found. This region is shared in all known homologues of Mad3p and Bub1p. This interaction places Mad3p at the effector end of the mitotic checkpoint where it may inhibit the activity of the APC by binding to Cdc20p. This indeed has been shown in other organisms. Sudakin *et al* purified a complex from HeLa cells which was able to inhibit the APC *in vitro* (Sudakin, Chan et al. 2001). This mitotic checkpoint complex (MCC), also contained hBub3, hCdc20 and hMad2. Mad2 has already been shown to inhibit the APC by others (Li, Gorbea et al. 1997; Fang, Yu et al. 1998; Kallio, Weinstein et al. 1998). However, the MCC was able to inhibit the APC 3,000-fold greater than Mad2 alone. A second group isolated Bub3 and BubR1 from human cells and found that this complex was able to inhibit the APC in the absence of Mad2 (Tang, Bharadwaj et al. 2001). Chapter 4 of this work presents data showing that the association between Mad3p and Cdc20p was dependent upon the presence of Mad2p in cells. It is possible that some Mad2 remained in the complex purified by Tang *et al* allowing the inhibition of the APC although BubR1 can indeed inhibit the APC independently of Mad2 at least *in vitro* (Fang 2002). If yeast Mad3p was able to inhibit the APC, and in so doing arrest mitosis, then the over-expression of Mad3p would cause a metaphase arrest. This was not seen in this work when cells over-expressing *MAD3* when examined under a microscope (Section 3.3.4) or when *MAD3* was over-expressed in the chromosome loss assay strain (Chapter 6). This may suggest a difference in the activity of budding yeast Mad3p and that of Mad3 in higher eukaryotes.

7.4 Tension Vs Attachment

The role of Mad3p in the checkpoint is still up for debate and although this thesis goes some way in identifying possible avenues for further research it does not address one dichotomy of mitotic checkpoint function; that is, does the checkpoint detect a lack of tension between the unattached kinetochore and the spindle pole or does it detect the unattached kinetochore itself?

Initial work using insect germ cells suggested that tension was the overriding signal. These mantid sperm cells were unusual in that they have two X chromosomes but only one Y chromosome resulting in many occasions when one of the X chromosomes is attached to only one pole of the mitotic spindle. Cells with this monopolar attachment arrest. This arrest is abolished when the unattached X chromosome is pulled by a micromanipulating needle thought to be applying tension to the unattached kinetochore (Li and Nicklas 1997). Tension had already been thought to be a signal for the checkpoint but this work strengthened the argument (Nicklas, Ward et al. 1995). Rieder *et al* did early work on establishing unattachment of the kinetochore as a signal for the checkpoint (Rieder, Schultz et al. 1994). The tension/attachment discussion has continued and today it is clear that the mitotic checkpoint can detect both tension and attachment (Zhou, Yao et al. 2002).

At present the debate surrounds which components of the checkpoint respond to unattached kinetochores and which respond to those not under tension from the poles. Mad2 was found to localise to kinetochores which were not attached to a spindle pole (Chen, Waters et al. 1996; Li and Benezra 1996). Another experiment went further and found that not only did Mad2 respond to attachment but it also did not respond to tension (Waters, Chen et al. 1998). Recent work has shown that the mitotic checkpoint may have two arms, each responding to either attachment or tension. Skoufias *et al* added a microtubule stabilising agent to HeLa cells called vinblastin. In low concentrations of vinblastin the kinetchores are attached to the spindle poles but they are not under tension. At these low concentrations Bub1 and BubR1 were recruited to the kinetochore, Mad2 is not. In higher concentrations when the microtubule-kinetochore attachment is disrupted Mad2 is also recruited to the

kinetochores (Skoufias, Andreassen et al. 2001). Therefore Bub1 and BubR1 respond to an absence of tension and only when the spindle is structurally compromised, resulting in free kinetochores, does Mad2 then associate with the kinetochore.

What mechanisms allow Bub1/BubR1 and Mad2 to respond to different signals? Mad2 closely associates with Mad1 (Chen, Brady et al. 1999; Luo, Tang et al. 2002). BubR1 interacts with CENP-E (Chan, Jablonski et al. 1999; Yao, Abrieu et al. 2000; Chen 2002). No interactions have been found between Mad2 and CENP-E or between BubR1 and Mad1. Cdc20 is the link between Mad2 and BubR1 that allows both to inhibit anaphase, could these separate interactions be the methods by which they respond to different vagaries during mitosis? The Ipl1p discussed in section 1.1.4 has now been implicated in the checkpoint response and may have a role in the Mad3p response to tension. One role of the Ipl1p kinase is in the bi-orentation of kinetochores during mitosis in budding yeast. The second role of Ipl1p kinase, its role in the spindle checkpoint, is a very recent development and is still under scrutiny by the checkpoint community.

The main evidence in support of Ipl1p role in the checkpoint suggests that it is responding to a lack of tension. Tension was recently found to be detected in budding yeast (Stern and Murray 2001). Biggins *et al* used two methods to produce kinetochores which were attached to the spindle assembly but were not under tension to show that Ipl1p was required to arrest the cell cycle in these circumstances (Biggins and Murray 2001). The recent work of two laboratories have strengthened the role of Ipl1p in detecting tension by performing experiments in mammalian systems using different chemical inhibitors of Aurora kinase (the mammalian homologue of Ipl1p) (Ditchfield, Johnson et al. 2003; Hauf, Cole et al. 2003). Together these papers point to Ipl1p/Aurora kinase having a role in the detection of tension in the spindle checkpoint although one paper has suggested a role in detecting attachment of chromosomes in fission yeast (Petersen and Hagan 2003). Although there is some argument where Ipl1/Aurora kinase functions in the mitotic checkpoint the evidence supporting its inclusion in the mitotic checkpoint is mounting. Recent evidence shows that the checkpoint component Mad3p (the focus of this work) is phosphorylated *in vitro* by Ipl1p kinase (Emma King, personal communication). This interaction, if proved correct, may be a link between BubR1/Mad3p and a mechanism for monitoring tension. Ipl1p has an opposing phosphatase in the form of Glc7p, a member of the PP1

family of phosphatases (Francisco, Wang et al. 1994). One of the more promising results from the two-hybrid assay described in chapter 5 of this work was to find a regulatory subunit of the PP2A phosphatase, Rts1p. As Mad3p may be phosphorylated during checkpoint activation (Emma King, per comm) this interaction with Rts1p may lead to the dephosphorylation of Mad3p and silencing of the checkpoint when the kinetochores are under tension and ready to be segregated into the daughter cells.

7.5 Bub1p and Mad3p share domains of homology but distinct roles

Chapter 4 identifies binding partners of Mad3p through two domains shared by Bub1p and Mad3p. Both Mad3p and Bub1p associate with Bub3p constitutively (this work and (Brady and Hardwick 2000)). The purpose of these complexes in mammalian cells is at least to localise Bub1 and BubR1 to the kinetochores of unattached sister chromatids (Basu, Logarinho et al. 1998; Taylor, Ha et al. 1998). The importance of the Bub1p/Bub3p complex is evident when one measures the chromosome loss rates of checkpoint mutants (Chapter 6). Why is the Mad3p/Bub3p complex not so important for chromosome stability? Mad3p and Bub1p may be involved in different processes, both relating to the checkpoint but with Bub1p having other kinetochore functions. Bub1p may have an essential role in mitosis such as the timing role already postulated (Taylor and McKeon 1997).

Mad3p binds to Bub3p through a conserved domain shared with Bub1p. Mad3p also binds to a known target of the mitotic checkpoint, Cdc20p, forming a quaternary complex with Mad2p, Cdc20p and Bub3p (Chapter 4). Mad3p itself was not shown to be modified during the cell cycle in this work but the Mad3p/Mad2p/Cdc20p/Bub3p complex was found to be most abundant in cells where the checkpoint is activated. As discussed in section 7.3 this association with Cdc20p and the possible interaction with Ipl1p place Mad3p in an effector role within the checkpoint where it can inhibit anaphase.

Bub1p has not been found to have this role. Bub1p binds to Mad1p in yeast cells and this association is most abundant in nocodazole arrested cells (Brady and Hardwick 2000). This interaction with Mad1p is essential for checkpoint function as a

mutation in Mad1p abolishing binding of Bub1p and Mad1p make cells benomyl sensitive (Brady and Hardwick 2000). The function of this first region of homology between Bub1p and Mad3p in the Bub1p protein is still not clear. One possible answer may come from a screen using this region of Bub1p to find interacting proteins. This is indeed underway in this laboratory although the success of this screen is uncertain as a screen of the entire yeast genome gave no sensible results for Bub1p (Uetz, Giot et al. 2000). Biochemical analysis has found an interaction between Bub1p and Skp1p (Kitagawa, Abdulle et al. 2003). Skp1p is a component of the CBF3 complex of the inner kinetochore (Table 1.1). This interaction and a genetic interaction with a putative component of the yeast kinetochore, Plc1p, strengthens a role for Bub1p in the structure of the kinetochore itself and not only its regulation (DeLillo, Romero et al. 2003). The association with the inner kinetochore, and the complex formed with Mad1p/Bub3p, place Bub1p in a good position to sense changes in kinetochore behaviour and via its kinase activity relay this message to the cell cycle machinery thereby acting as a sensor and/or transducer in the classical checkpoint model (Section 1.2). As discussed Mad3p may have an effector role in the checkpoint.

7.6 Mad3p and disease

Section 1.2.4 presents a case for the involvement of the mitotic checkpoint in disease. This work adds to this by showing an effect on the stability of chromosomes in cells with a deletion in the reading frames of the spindle checkpoint (Chapter 6). The loss rates of the heterozygous strains described in section 6.2.4 shows a relationship between the amount of checkpoint protein in the cell and the stability of the genome. This is borne out by the relative loss rates of the homozygous *mad* mutant having a higher loss rate than the heterozygous and the heterozygous strain having a higher loss rate that the wild type *MAD* homozygous diploid.

It is clear from the work in chapter 6 that the *BUB1* and *BUB3* genes are most important for the stability of chromosomes given the high rates of loss in their mutant strains. Indeed, a mutation in a somatic copy of hBub1 has been found in lung cancer primary cells (Gemma, Seike et al. 2000).

The exact role of the mitotic checkpoint in the progression of disease in humans is still unsure but with this work does suggest Bub1p and Bub3p may have an important role in preventing aneuploidy.

7.7 A model for mitotic checkpoint function

Taken together it is possible to construct a model of mitotic checkpoint function in budding yeast. Figure 7.1 shows the potential roles of the components at the kinetochore. The constitutive complexes of Bub1p/Bub3p and Mad3p/Bub3p are localised to the unattached kinetochore upon mitotic entry (during S-Phase in budding yeast). The Mad1p/Mad2p complex also localises to the unattached kinetochore (Jin, Spencer et al. 1998). This places Mad2p and Mad3p proteins in close proximity allowing the formation of the Mad2p/Mad3p/Cdc20p complex. As discussed in section 7.3 it is fair to postulate that Mad3p has an effector role in the checkpoint, inhibiting the APC perhaps via an interaction with the Ipl1p kinase. The composition of the Bub1p/Mad1p complex suggests a signally role for Bub1p within the mitotic checkpoint although it has to be noted that the interaction between Mad1 and Bub1 has not been seen in mammalian cells (Burke 2000). Figure 7.1 attempts to formulate this evidence into a suitable model for checkpoint function.

Further questions must be answered before the mitotic checkpoint is finally understood. In particular the question of activation of the checkpoint is one. We now know that tension and attachment can activate the checkpoint but what molecular changes occur within the kinetochore to allow this? Activation of the checkpoint is one question another and much less understood challenge is the deactivation of the checkpoint once the conditions for anaphase have been met. What silences Mad3 and Mad2 allowing the progress of anaphase? These and many other questions will be answered in the years to come and no doubt, more enigmas will be unearthed in the process.

Figure 7.1

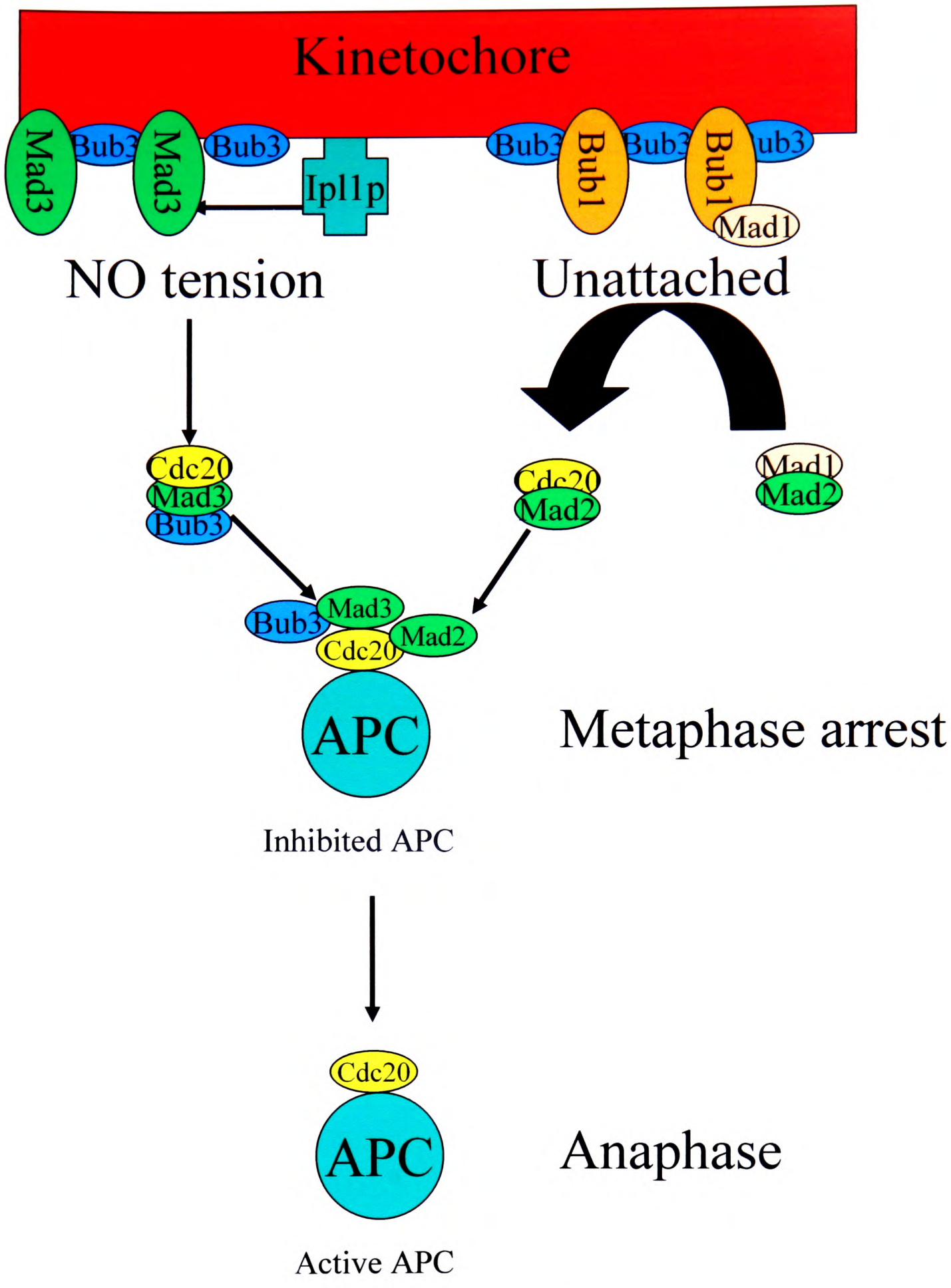


Figure 7.1 A model of mitotic checkpoint function. Mad3 is somehow stimulated to respond to a lack of tension exerted on a kinetochore, possibly by an interaction with Ipl1p kinase. It then associates with Cdc20 and inhibits the APC. Simultaneously the Mad1/Mad2 complex is localised to the kinetochore of an unattached sister chromatid where it too associates with Cdc20 allowing the inhibition of the APC. The Bub1/Bub3/Mad1 complex forms perhaps to localise Mad2 to these kinetochores and to amplify the signal throughout the cell.

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